The chloroplast-to-chromoplast transition in tomato fruit

Wanping Bian

Université de Toulouse

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Directeur(s) de Thèse :
Jean-Claude PECH
Christian CHERVIN

Rapporteurs :

Christian CHEVALIER, DR INRA Bordeaux
Rebecca STEVENS, CR1 INRA Avignon

Membre(s) du jury :

Christian CHERVIN, PR INP-ENSAT
Jean-Claude PECH, PR INP-ENSAT
Rebecca STEVENS, CR1 INRA Avignon
Christian CHEVALIER, DR INRA Bordeaux
Mondher BOUZAYEN, PR INP-ENSAT
Zhengguo LI, PR Univ. Chongqing, China

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Publications

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CONTENT

Résumé

L'un des phénomènes les plus importants survenus pendant la maturation du fruit de tomate est le changement de couleur du vert au rouge. Ce changement a lieu dans les plastes et correspond à la différenciation des plastes photosynthétiques, les chloroplastes, en plastes non-photosynthétiques qui accumulent des caroténoïdes, les chromoplastes. Dans cette thèse, nous présentons d'abord une introduction bibliographique sur le domaine de la transition chloroplaste-chromoplaste, en décrivant les modifications structurales et physiologiques qui se produisent pendant la transition. Puis, dans le premier chapitre, nous présentons des observations microscopiques de plastes isolés à trois stades de mûrissement, puis des enregistrements en temps réel de la fluorescence des pigments sur les tranches de fruits de tomate. Il a été possible de montrer que la transition chloroplaste-chromoplaste était synchrone pour tous les plastes d'une seule cellule et que tous les chromoplastes proviennent de chloroplastes préexistants. Dans le deuxième chapitre, une approche protéomique quantitative de la transition chloroplaste-chromoplaste est présentée, pour identifier les protéines différentiellement exprimées. Le traitement des données a identifié 1932 protéines parmi lesquelles 1529 ont été quantifiées par spectrométrie de masse. Les procédures de quantification ont ensuite été validées par WESTERN blot de certaines protéines. La chromoplastogénèse comprend les changements métaboliques suivants : diminution de l'abondance des protéines de réaction à la lumière et du métabolisme des glucides, et l'augmentation de la biosynthèse des terpénoïdes et des protéines de stress. Ces changements sont couplés à la rupture de la biogenèse des thylakoïdes, des photosystèmes et des composants de production d'énergie, et l'arrêt de la division des plastes. Dans le dernier chapitre nous avons utilisé la lincomycine, un inhibiteur spécifique de la traduction à l'intérieur des plastes, afin d'étudier les effets sur la maturation des fruits et sur l'expression de gènes nucléaires impliqués dans la maturation. Les résultats préliminaires indiquent que l'inhibition de la traduction des protéines dans les plastes affecte la maturation du fruit en réduisant l'accumulation de caroténoïdes. L'expression de plusieurs gènes nucléaires a été modifiée mais une relation claire avec le phénotype altéré de maturation n'a pas pu être établie.

Au total, notre travail donne de nouveaux aperçus sur le processus de différenciation chromoplaste et fournit des données nouvelles ressources sur le protéome plaste.

Abstract

One of the most important phenomenons occurring during tomato fruit ripening is the color change from green to red. This change takes place in the plastids and corresponds to the differentiation of photosynthetic plastids, chloroplasts, into non photosynthetic plastids that accumulate carotenoids, chromoplasts. In this thesis we first present a bibliographic introduction reviewing the state of the art in the field of chloroplast to chromoplast transition and describing the structural and physiological changes occurring during the transition. Then, in the first chapter we present an *in situ* real-time recording of pigment fluorescence on live tomato fruit slices at three ripening stages. By viewing individual plastids it was possible to show that the chloroplast to chromoplast transition was synchronous for all plastids of a single cell and that all chromoplasts derived from pre-existing chloroplasts. In chapter two, a quantitative proteomic approach of the chloroplast-to-chromoplast transition is presented that identifies differentially expressed proteins. Stringent curation and processing of the data identified 1932 proteins among which 1529 were quantified by spectral counting. The quantification procedures have been subsequently validated by immune-blot evaluation of some proteins. Chromoplastogenesis appears to comprise major metabolic shifts (decrease in abundance of proteins of light reactions and carbohydrate metabolism and increase in terpenoid biosynthesis and stress-related protein) that are coupled to the disruption of the thylakoid and photosystems biogenesis machinery, elevated energy production components and loss of plastid division machinery. In the last chapter, we have used lincomycin, a specific inhibitor of protein translation within the plastids, in order to study the effects on fruit ripening and on the expression of some ripening-related nuclear genes. Preliminary results indicate that inhibiting protein translation in the plastids affects fruit ripening by reducing the accumulation of carotenoids. The expression of several nuclear genes has been affected but a clear relationship with the altered ripening phenotype could not be established.

Altogether, our work gives new insights on the chromoplast differentiation process and provides novel resource data on the plastid proteome.

摘要

番茄果皮由绿色到红色的转变是发生在其成熟过程中的最重要的现象之一。这种变化的本质是番茄果实中的质体发生了变化。伴随着可进行光合作用的叶绿体到非光合作用的色质体的转变，大量类胡萝卜素同时也积累在了色质体中，从而使得果实颜色由绿色变为红色。在本文中，我们综合介绍了叶绿体到色质体转变这一领域的最新研究进展，并且详细的描述了在这一转变过程中质体在结构上，生理上以及分子方面的变化。接下来在第一章中，我们利用激光共聚焦显微镜原位、实时的记录了活体番茄切片在三个不同阶段中所含色素荧光的变化。并且通过对不同时期单个质体的显微镜的观察，我们认为单个细胞的所有质体在从叶绿体转变到色质体的过程中很有可能是同步的，而且所有色质体都是由之前存在的叶绿体转变来的。在第二章中，用定量蛋白组学的方法研究叶绿体到色质体转变过程中不同蛋白的表达丰度。经过严格校对，有 1932 个蛋白质被鉴定了出来，其中 1529 个经过光谱计数定量。并且对其中一些蛋白的量化结果用免疫杂交的方法进行了验证。色质体产生的过程中包含了一系列重要代谢反应中相关蛋白的变化（例如，光反应和碳水化合物代谢有关的蛋白质在数量上减少了，但是萜类物质生物合成和外界压力应答相关的蛋白却增加了），所有这些变化都伴随着类囊体和光合系统机器的破坏，能量物质相关产物的增加，以及质粒分裂系统的消失。在最后一章中，我们利用林可霉素可以特异的抑制质体中蛋白质的翻译这一特性来研究水果成熟过程中细胞核成熟相关基因的表达。初步结果显示，通过抑制质体蛋白的合成，间接影响到了水果中类胡萝卜素的积累，从而影响到了水果的成熟。同时一些其它的核基因的表达也受到了影响，但是它们与果实成熟的关系还需要进一步的研究。

综上所述，本工作为色质体的形成提供了新的观点并且为质体蛋白组学提供的新的数据资源。

Objectif de la thèse

La maturation des fruits est un processus de développement subtilement orchestré, unique pour les plantes, qui se traduit par d'importants changements physiologiques et métaboliques, conduisant finalement à la sénescence des fruits et la dispersion des graines (Pirrello et al., 2009). Dans de nombreux fruits, l'un des changements les plus importants et les plus visibles au cours de la maturation correspond à la perte de chlorophylle et la synthèse de composés colorés tels que des caroténoïdes. Ce processus se déroule au niveau sub-cellulaire dans le plaste. Le plaste est soumis à d'importantes modifications structurales et biochimiques au cours du développement des plantes, ce qui reflète l'état physiologique de la cellule. Par conséquent, plusieurs types de plastes (souvent interconvertibles) ont été décrits avec des fonctions spécialisées. Parmi eux, les chloroplastes abritent des fonctions essentielles telles que la photosynthèse, synthèses de glucides, lipides, isoprénoïdes (caroténoïdes, les quinones ...). Il est maintenant clair que les plastes non-verts, bien que dépourvu de la capacité photosynthétique, sont des formes métaboliquement actives de plastes, souvent impliqués dans la biosynthèse de nombreux composés. Cela est vrai pour chromoplastes qui sont souvent formés à partir de la différenciation des chloroplastes et défini comme plastes dépourvus de chlorophylle, qui accumulent des pigments de la classe des caroténoïdes (Marano et al., 1993; Camara et al., 1995). Ces derniers composés donnent leur couleur distinctive. Les chromoplastes sont présents dans certaines fleurs et de fruits, et parfois dans les racines et les feuilles. Dans les fleurs et le fruit, ils servent la stratégie de reproduction de la plante en attirant les pollinisateurs ou les animaux qui dispersent les graines.

La division des plastes est associée à la division des cellules végétales. Ils se distinguent d'autres types de plastes dans différents types de cellules végétales. Il y a plusieurs centaines de chromoplastes dans les cellules mûres de fruits de tomate. L'augmentation de la population de plastes a lieu pendant le développement du fruit vert, ce qui entraîne de grandes populations de chloroplastes, qui vont ensuite se différencier en chromoplastes (Cookson et al., 2003). Les changements structurels au cours de la transition du chloroplaste-chromoplaste ont été largement étudiés par microscopie

électronique de la tomate (Rosso, 1968; Harris et Spurr, 1969) et du poivron (Spurr et Harris, 1968) et aussi par la fluorescence de la GFP ciblée pour les composés plastidiaux (Pyke, 2007). Gunning (2005) a fait des images en couleur en microscopie en champ clair des plastes où les chromoplastes apparaissent comme rouge foncé ou orange. Cependant, il n'y a pas d'observation simultanée de chlorophylles et de caroténoïdes sur plaste isolé lors des étapes de maturation de la tomate. Nous avons effectué l'observation à l'aide de microscopie confocale à balayage laser pour observer la perte de chlorophylles et de l'accumulation des caroténoïdes dans trois différents stades de différenciation des plastes: chloroplastes (à partir de fruits mûrs-verts), plastes intermédiaire (à partir de fruits breaker) et chromoplastes (fruits mûrs). Un dispositif microscopique a également permis de filmer les changements de fluorescence au niveau cellulaire sur tranches de tomate, permettant de révéler la dynamique de la transition du chloroplaste-chromoplaste.

Les voies métaboliques individuelles ont été largement étudiées dans les chromoplastes. Il a été montré que chromoplastes sont actifs dans le métabolisme des glucides, pour répondre aux exigences des différentes activités biosynthétiques qui opèrent dans ces plastes, par exemple la biosynthèse des caroténoïdes (Fraser et al., 1994; Bramley, 2002), et les métabolismes lipidiques (Liedvogel and Kleinig, 1977; Li-Beisson et al., 2010), probablement pour recycler les lipides des thylakoïdes et reconstruire de nouvelles membranes non chlorophylliennes. D'autres activités, telles que celles impliquées dans la synthèse de la cystéine (Romer et al., 1992), du glutathion (Mittova et al., 2003; Marti et al., 2009), des tocophérols (Arango et Heise, 1998; Dellapenna et Pogson, 2006), sont également présentes dans les chromoplastes. Des synthèses bibliographiques spécifiquement dédiées à la biogenèse et l'activité métabolique des chromoplastes ont été publiées (Ljubesic et al., 1991;. Bouvier et Camara, 2006). Certaines informations peuvent également être trouvés dans les documents consacrés à la différenciation des plastes en général (Pyke, 2007).

Ces dernières années, des technologies à haut débit sont apparues et ont commencé à être appliquées à l'étude du métabolisme des plastes. Par exemple, les approches transcriptomique et la protéomique ont donné des informations inédites sur les aspects biochimiques et moléculaires de la différenciation des chromoplastes en relation avec

l'activité transcriptionnelle et traductionnelle du génome plastidial (Kahlau et Bock, 2008; Kleine Leister, 2011). Ils ont fourni une vue systématique de l'expression des gènes du génome pastidial au cours de la différenciation du chromoplaste dans la tomate. Toutefois, le génome de plaste est très faible de sorte que la plupart des protéines plastidiales sont codées par le génome nucléaire. Dans ces conditions, le protéome plastidial comprend plus de 90% de protéines importées. Le protéome plastidial des plantes supérieures a été étudié principalement sur le chloroplaste (van Wijk et Baginske, 2011). Moins d'informations sont disponibles sur le protéome du chromoplaste et peu d'études ont été réalisées sur les fruits, comme le poivron, la tomate et l'orange. Siddique et al. (2006) identifié 151 protéines du poivron, grâce à une nouvelle stratégie pour l'identification de base de données indépendante des protéines ce qui donne un aperçu des voies métaboliques majeures actives dans le chromoplaste. Barsan et al. (2010) ont analysé le protéome de la tomate fruits chromoplaste rouge et ont révélé la présence de 988 protéines parmi lesquelles 209 protéines n'étaient pas mentionnées dans des banques de données plastidiales. La combinaison des données physiologiques et protéomique a fourni de nouveaux détails sur les métabolismes des chromoplastes de tomates. Un autre travail effectué sur les oranges par Zeng et al. (2011) a permis d'identifier 493 protéines dont 418 protéines plastidiales. Une comparaison avec les données de protéomique de chromoplastes de tomates suggère un niveau élevé de similitude dans de nombreuses voies métaboliques. Jusqu'à présent, ces études ont été réalisées sur des plastes typiques tels que les chloroplastes et chromoplastes, mais le processus de différenciation survenant au cours de la transition du chloroplaste-à-chromoplaste n'a pas été abordé par des approches protéomiques. Dans cette thèse, nous avons entrepris une caractérisation en profondeur de la différenciation du chromoplaste par des techniques de protéomique appliquée à trois stades différents de différenciation des plastes de tomates lors de la transition du fruit vert au fruit rouge. L'objectif était de quantifier les changements dans l'abondance des protéines afin de caractériser les changements majeurs d'activité métabolique et des processus de régulation.

De nombreux gènes nucléaires et plastidiaux impliqués dans la transition du chloroplaste-chromoplaste agissent comme des régulateurs pour contrôler ce processus. La coordination de l'expression des gènes plastidiaux et nucléaires joue un rôle essentiel

lors de la différenciation des plastes. Par exemple, pour la biosynthèse de pigments, l'expression des gènes nucléaires est nécessaire (Rüdiger et Grimm, 2006). Des études approfondies ont été réalisées sur la signalisation entre le noyau et les plastes, plus particulièrement pour l'expression de gènes plastidiaux corrélés à la photosynthèse (Leon et al., 1998). Afin de découvrir les effets de l'expression du génome plastidial sur les gènes codés par le noyau, certains auteurs ont inhibé la traduction dans les plastes par un inhibiteur spécifique, la lincomycine (Sullivan et Gray, 1999, 2002;. Hideg et al., 2007). Il a été constaté que l'expression de gènes nucléaires associés à la photosynthèse dans les chloroplastes (PhANGs) ont été réprimés lors d'un traitement à la lincomycine montrant qu'il y a des signaux « rétrogrades » du plaste vers le noyau (Oelmuller et al, 1986;. Mulo et al., 2003;. Dietzel et al., 2008). Un traitement à la lincomycine de semis de tabac de 7 jours, la transcription des complexes collecteurs de lumière (Lhcb1) a été supprimée alors qu'aucun effet n'a été observé sur la transcription de gènes nucléaires codant pour des protéines mitochondrial (ATP2) ou cytosoliques (actine) (Gray et al., 2003). L'objectif de notre travail consiste à évaluer si l'inhibition de la traduction des protéines à l'intérieur des plastes a un effet sur les processus de maturation des fruits et sur l'expression de gènes nucléaires. Ceci permettra d'impliquer ou non la présence d'une signalisation rétrograde pendant la différentiation du chromoplaste dans le fruit de tomate.

En résumé, la transition du chloroplaste-chromoplaste est un processus très complexe comprenant de nombreux événements moléculaires et biochimiques et des changements dans la structure interne. Notre thèse débutera par une synthèse des événements métaboliques et moléculaires qui ont été décrits jusqu'à présent au cours de la biogenèse des chromoplastes. Puis, dans un premier chapitre, une étude par microscopie confocale à balayage laser est présentée, montrant comment les chloroplastes deviennent des chromoplastes, par l'observation des pigments dans les plastides isolés et un film sur des cellules de tranches de fruits. Dans un deuxième chapitre, une analyse protéomique quantitative a été réalisée dans le but de comprendre la régulation des changements métaboliques et structurels qui se produisent dans les plastes de fruits de tomate lors du passage de chloroplaste à chromoplaste. Dans un troisième chapitre, la lincomycine, a été utilisée pour inhiber la traduction des protéines dans le plaste afin d'étudier la régulation de l'expression génique au cours de la transition du chloroplaste-chromoplaste.

Objective of the Thesis

Fruit ripening is a sophisticatedly orchestrated developmental process, unique to plants, that results in major physiological and metabolic changes, ultimately leading to fruit decay and seed dispersal (Pirrello, 2009). In many fruit, one of the most important and more visible changes during ripening corresponds to the loss of chlorophyll and the synthesis of colored compounds such as carotenoids. This process takes place at the sub-cellular level in the plastid. The plastid is subject to considerable structural and biochemical changes during plant development, reflecting the physiological state of the cell. Consequently, several types of plastids (often interconvertible) with specialised functions have been described. Among them, chloroplasts harbor essential functions such as photosynthesis, carbohydrate, lipid, isoprenoid (carotenoids, quinones…) metabolisms, etc. It is now clear that non-green plastids, although devoid of the photosynthetic capability, are metabolically active forms of plastids, often involved in the biosynthesis of many compounds. This is true for chromoplasts which are often formed from the differentiation of chloroplasts and defined as plastids lacking chlorophylls, which accumulate pigments of the carotenoid class (Marano et al., 1993; Camara et al., 1995). The latter compounds give them their distinctive color. Chromoplasts are present in some flowers and fruit, and occasionally in roots and leaves. In flower and fruit, they serve the reproduction strategy of the plant by attracting pollinators or animals that will disperse the seeds.

Plastids division is associated with plant cells division for remaining resident in the plant cell. They are differentiated to other types of plastids in different types of plant cell. The chromoplast population in ripe tomato fruit cells reaches several hundreds. Most of the plastids population increase takes place during the development of the green fruit, resulting in large chloroplast populations, which then differentiate into chromoplasts (Cookson et al., 2003). The structural changes during the chloroplast-to-chromoplast transition have been extensively studied by electron microscopy in tomato (Rosso, 1968; Harris and Spurr, 1969) and in bell pepper (Spurr and Harris, 1968) and also by fluorescence of GFP targeted to the plastids compounds (Pyke, 2007). Gunning (2005)

has made color images under bright field microscopy of plastids where chromoplasts appear as dark red or orange. However, there is no observation of chlorophyll and carotenoid fluorescence on the single isolated plastid from three different stages of tomato fruit. We performed the observation using a laser scanning confocal microscopy to monitor the loss of chlorophyll and accumulation of the carotenoid with three different stages of plastid differentiation: chloroplast (from mature-green fruit), intermediate plastid (from breaker fruit) and chromoplast (from ripe fruit). A time-lapse recording was performed to analyse the fluorescence of live tomato tissue slices to reveal the dynamic of the chloroplast-chromoplast transition.

Individual metabolic pathways have been extensively studied in the chromoplasts. It has been shown that chromoplasts are active in carbohydrate metabolism, most likely to sustain the requirements for the different biosynthetic activities operating in these plastids (e.g. carotenoid biosynthesis,), and in acyl lipid metabolism (Liedvogel and Kleinig, 1977; Li-Beisson et al., 2010), most likely to recycle the lipids from the disappearing thylakoids and to rebuild new achlorophyllous membranes. Other activities, such as those involved in the synthesis of cysteine (Romer et al., 1992), glutathione (Mittova et al., 2003; Marti et al., 2009), tocopherols (Arango and Heise, 1998; DellaPenna and Pogson, 2006), are also found in chromoplasts. Reviews specifically dedicated to the biogenesis and metabolic activity of chromoplasts have been published (Ljubesic et al., 1991; Bouvier and Camara, 2006). Some information can also be found in papers dedicated to plastid differentiation in general (Pyke, 2007).

In the recent years, high-throughput technologies have emerged that have started to be applied to the study of plastids metabolism. For instance, transcriptomics and proteomics approaches have given novel information of the biochemical and molecular aspects of the differentiation of chromoplasts in relation with the transcriptional and translational activity of the plastid genome (Kahlau and Bock, 2008; Kleine and Leister, 2011). They provided a systematic view of the expression of genes of the plastid genome during chromoplast differentiation in tomato. However, the plastid genome is very small so that most of the plastid proteins are encoded by the nuclear genome. In these conditions, the plastid proteome comprises more than 90% of imported proteins. The

whole plastid proteome of higher plants has been studied with strong emphasis on the chloroplast (van Wijk and Baginsky, 2011). Less information is available on the chromoplast proteome with few studies on fruits, such as pepper, tomato and sweet orange. Siddique et al. (2006) identified 151 proteins from pepper based on a novel strategy for the database-independent identification of proteins and provided an overview of the major metabolic pathway that active in chromoplast. (Barsan et al., 2010) have analysed the proteome of red tomato fruits chromoplast and revealed the presence of 988 proteins among which 209 proteins had not been listed in plastidial databanks. The combination of physiological and proteomics data have provided new insights into tomato chromoplast metabolic characteristics. Another work performed on sweet orange fruits by Zeng et al. (2011) has identified 493 proteins of which 418 are putative plastid proteins. A comparison with tomato chromoplast proteomics data suggested a high level of conservation in a broad range of metabolic pathways. So far these studies have been carried out on typical plastid structures such as chloroplasts and chromoplasts, but the differentiation process occurring during the chloroplast-to-chromoplast transition has not been addressed by proteomics approaches. In this thesis we have undertaken an in-depth characterization of the chromoplast differentiation by proteomics techniques applied at three different stages of tomato plastids differentiation during the ripening process from mature-green to red fruit. The objective was to quantify the changes in protein abundance so as to characterize the major shifts in metabolic activity and the accompanying regulatory processes.

Many of the nuclear and plastids genes involved in the transition of chloroplast-chromoplast act as regulators for controlling this process. The coordination between plastids and nucleus gene expression plays an essential role during plastids differentiation. For instance, for the biosynthesis of pigments, nuclear genes expression are required (Rüdiger and Grimm, 2006). Extensive studies have been performed on the nucleus to plastid signalling, more specifically for the expression of photosynthesis correlated genes in chloroplast (Leon et al., 1998). In order to uncover the effects of the expression of the plastid genome on nuclear-encoded genes, some authors have inhibited the translation in plastids by a specific protein translation inhibitor, lincomycin (Sullivan and Gray, 1999, 2002; Hideg et al., 2007). It has been found that photosynthesis-associated nuclear genes

of chloroplasts (PhANGs) were repressed upon lincomycin treatment giving support to retrograde signalling from the plastid to the nucleus (Oelmuller et al., 1986; Mulo et al., 2003; Dietzel et al., 2008). With lincomycin treatment of 7-day-old tobacco seedlings, transcripts of light-harvesting complexes (Lhcb1) were suppressed while no effect was observed on transcripts of nuclear genes encoding mitochondrial (Atp2) or cytosolic (Actin) proteins (Gray et al., 2003). The objective of our work is to evaluate whether the inhibition of protein translation within the plastids has an effect on the fruit ripening process and in the expression of nuclear genes. This would allow involving or not the presence of retrograde signalling during chromoplast differentiation in tomato fruit.

In summary, the chloroplast-to-chromoplast transition is a very complex process comprising numerous molecular and biochemical events and changes in internal structure. Our thesis will start with a review of the metabolic and molecular events that have been described so far during the biogenesis of chromoplasts. Then, in a first chapter, a laser scanning confocal microscopy will be presented, showing how all chloroplasts become chromoplasts, by observation of pigments in isolated plastids and real-time recording of fruit slice tissues. In a second chapter, a quantitative proteomic analysis has been carried out in order to understand the regulation of the metabolic and structural changes occurring in tomato fruit plastids during the transition from chloroplast to chromoplast. In a third chapter, the antibiotic lincomycin, an inhibitor of plastid translation, was used to inhibit translation of chromoplast proteins so as to investigate the regulation of gene expression during chloroplast to chromoplast transition.

General Introduction

Fruit play an important role in nutrition for a healthy life of humans. Due to their importance, a large number of studies have been dedicated to the understanding of the ripening process and to the improvement of the organoleptic qualities of fruit in the search for fruit rich in aroma and beneficial nutrients with long shelf-life. The ripening process of fruit is a sophisticatedly orchestrated developmental process, unique to plants, that results in major physiological and metabolic changes, ultimately leading to seed dispersal (Pirrello, 2009).

Tomato is one of the most important plants for human diet. Wild tomato (*Solanum lycopersicum L.*) is native from the coastal plain to the foothills of the Andes of western South America (Peralta et al., 2005). Now, this species is grown worldwide from sea level to 4000 meters of altitude. It is well known that tomato is beneficial to health due to the abundance of antioxidants. The most important antioxidants present in tomato fruit are carotenes which are colored compounds giving tomatoes their characteristic color.

During ripening, the changes in color of the fruit represent one of the most important and complex event. They are used as obvious markers for fruit ripening and as important quality attributes. The color of the tomato is a major factor for the consumer's purchase decision too (Radzevicius et al., 2009). Depending on the genotype, tomato fruit develop different colors during the ripening process, including green, yellow and red. The external color of tomato fruit is the result of both flesh and skin color (Yahia and Brecht, 2012). The complexity of tomato color is due to the presence of a diversity of carotenoid pigments in different concentrations (López Camelo and Gómez, 2004). The most important pigments of ripening tomato fruit are chlorophyll, lycopene and beta-carotene, which accumulate concomitantly with the decrease in chlorophyll content during the transition from chloroplast to chromoplast (Fraser et al., 1994).

Chloroplasts and chromoplasts belong to the plastid class of sub-cellular organelles of endo-symbiotic origin that are present under various forms in plant and algae. They possess a wide range of metabolic pathways including nitrate assimilation, starch

metabolism, fatty acid biosynthesis… Chloroplasts represent one form of plastid especially devoted to photosynthesis in green plants and algae. Chromoplasts have the particularity to accumulate pigments in fruit and flowers. Profound morphological and metabolic changes happen during the transition from chloroplast to chromoplast that have been reviewed in recent papers from our laboratory to which I have participated as first co-author (Bian et al., 2011) or as co-author (Egea et al., 2010). After the present short general introduction a review of the literature published on the topic of chromoplast differentiation will be presented. It corresponds to the Bian et al paper.

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Review Article

Metabolic and Molecular Events Occurring during Chromoplast Biogenesis

Wanping Bian,[1,2] Cristina Barsan,[1,2] Isabel Egea,[1,2] Eduardo Purgatto,[3] Christian Chervin,[1,2] Mohamed Zouine,[1,2] Alain Latché,[1,2] Mondher Bouzayen,[1,2] and Jean-Claude Pech[1,2]

[1] *Génomique et Biotechnologie des Fruits, INP-ENSA Toulouse, Universite´de Toulouse, avenue de l'Agrobiopole BP 32607, Castanet-Tolosan 31326, France*
[2] *Génomique et Biotechnologie des Fruits, INRA, Chemin de Borde Rouge, Castanet-Tolosan 31326, France*
[3] *Departmento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Avenue Prof. Lineu Prestes 580, bl 14, 05508-000 São Paulo, SP, Brazil*

Correspondence should be addressed to Jean-Claude Pech, pech@ensat.fr

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Chromoplasts are nonphotosynthetic plastids that accumulate carotenoids. They derive from other plastid forms, mostly chloroplasts. The biochemical events responsible for the interconversion of one plastid form into another are poorly documented. However, thanks to transcriptomics and proteomics approaches, novel information is now available. Data of proteomic and biochemical analysis revealed the importance of lipid metabolism and carotenoids biosynthetic activities. The loss of photosynthetic activity was associated with the absence of the chlorophyll biosynthesis branch and the presence of proteins involved in chlorophyll degradation. Surprisingly, the entire set of Calvin cycle and of the oxidative pentose phosphate pathway persisted after the transition from chloroplast to chromoplast. The role of plastoglobules in the formation and organisation of carotenoid-containing structures and that of the *Or* gene in the control of chromoplastogenesis are reviewed. Finally, using transcriptomic data, an overview is given the expression pattern of a number of genes encoding plastid-located proteins during tomato fruit ripening.

1. Introduction

Chromoplasts are nonphotosynthetic plastids that accumulate carotenoids and give a bright colour to plant organs such as fruit, flowers, roots, and tubers. They derive from chloroplasts such as in ripening fruit [1], but they may also arise from proplastids such as in carrot roots [2] or from amyloplasts such as in saffron flowers [3] or tobacco floral nectaries [4]. Chromoplasts are variable in terms of morphology of the carotenoid-accumulating structures and the type of carotenoids [5, 6]. For instance, in tomato, lycopene is the major carotenoid, and it accumulates in membrane-shaped structures [7] while in red pepper beta-carotene is prominent and accumulates mostly in large globules [8]. Reviews specifically dedicated to the biogenesis of chromoplasts have been published [9–11]. Some information can also be found in papers dedicated to plastid differentiation in general [12, 13]. Thanks to transcriptomics and proteomics approaches, novel information is now available on the biochemical and molecular aspects of chromoplasts differentiation [14–16]. The present paper will review these novel data and provide a recent view of the metabolic and molecular events occurring during the biogenesis of chromoplasts and conferring specificities to the organelle. Focus will be made on the chloroplast to chromoplast transition.

2. Chromoplast Differentiation Is Associated with Important Structural, Metabolic, and Molecular Reorientations

Important structural changes occur during the chloroplast to chromoplast transition, thylakoid disintegration being the most significant (Figure 1). Early microscopic observations

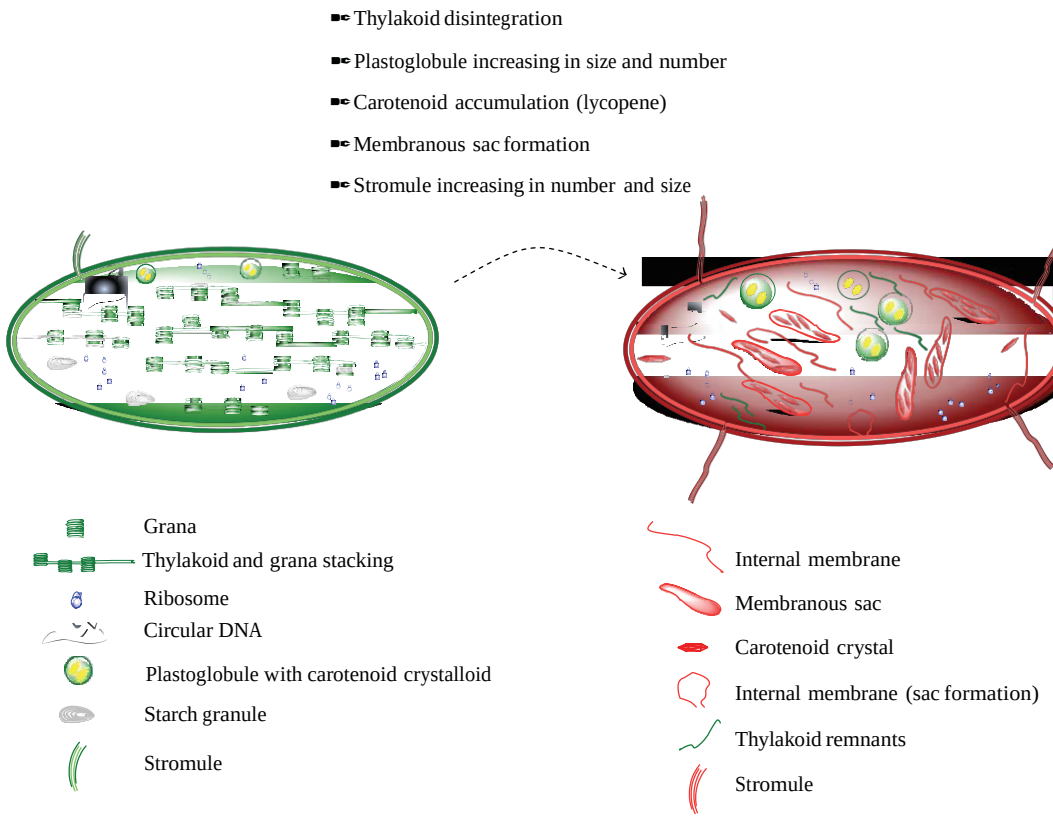


Figure 1: Schematic representation of the main structural changes occurring during the chloroplast to chromoplast transition.

have shown that plastoglobuli increase in size and number during the chloroplast-chromoplast transition [7] and that the internal membrane system is profoundly affected at the level of the grana and intergrana thylakoids [17]. Stromules (stroma-filled tubules) that are dynamic extensions of the plastid envelope allowing communication between plastids and other cell compartments like the nucleus [18] are also affected during chromoplastogenesis. A large number of long stromules can be found in mature chromoplasts contrasting with the few small stromules of the chloroplasts in green fruit [19]. It can therefore be assumed that the exchange of metabolites between the network of plastids and between the plastids and the cytosol is increased in the chromoplast as compared to the chloroplast. However, the most visible structural change is the disruption of the thylakoid grana, the disappearance of chlorophyll, and the biogenesis of carotenoid-containing bodies. Associated with the structural changes, the toc/tic transmembrane transport machinery is disintegrated [16, 20]. The noncanonical signal peptide transport [21] and intracellular vesicular transport [22, 23] may represent the most active form of trans-membrane transport into the chromoplast as compared to the chloroplast. Proteins involved in vesicular transport were detected in the tomato chromoplastic proteome [16].

One of the most visible metabolic changes occurring during the chloroplast to chromoplast transition is the loss of chlorophyll and the accumulation of carotenoids [24]. A spectral confocal microscopy analysis of carotenoids and chlorophylls has been carried out during the chloroplast to chromoplast transition in tomato fruit, including a time-lapse recording on intact live tissue [25]. Details of the early steps of tomato chromoplast biogenesis from chloroplasts are provided at the cellular level that show the formation of intermediate plastids containing both carotenoids and chlorophylls. This study also demonstrated that the chloroplast to chromoplast transition was synchronous for all plastids of a single cell and that all chromoplasts derived from preexisting chloroplasts.

The photosynthetic machinery is strongly disrupted and a reduction in the levels of proteins and mRNAs associated with photosynthesis was observed [26]. Also the decrease in photosynthetic capacity during the later stages of tomato fruit development was confirmed by transcriptomic data [27]. However, part of the machinery persist in the chromoplast. It has been suggested that it participates in the production of C4 acids, in particular malate a key substrate for respiration during fruit ripening [28]. In the tomato chromoplast proteome, all proteins of the chlorophyll biosynthesis branch are lacking [16]. In the early stages of tomato fruit ripening, the fruits are green and the plastids contain low levels of carotenoids that are essentially the same as in green leaves, that is, mainly β-carotene, lutein, and violaxanthin. At the "breaker" stage of ripening, lycopene begins to accumulate and its concentration increases 500-fold in ripe fruits, reaching *ca.* 70 mg/g fresh weight [24]. During the ripening of tomato fruit, an upregulation of

the transcription of *Psy* and *Pds*, which encode phytoene synthase and phytoene desaturase, respectively, was reported [29]. One of the main components of the carotenoid-protein complex, a chromoplast-specific 35-kD protein (chrC), has been purified and characterized in *Cucumis sativus* corollas. It showed increasing steady-state level in parallel with flower development and carotenoid accumulation, with a maximum in mature flowers [30]. In tomato, concomitantly with increased biosynthesis of lycopene, the processes for splitting into β and γ carotene were absent [16]. The mRNAs of *CrtL-b* and *CrtL-e* were strongly downregulated during fruit ripening [29]. They encode lycopene β-cyclase and ε-cyclase, enzymes involved in the cyclization of lycopene leading to the formation of β and δ carotene, respectively. In these conditions, the low rate of cyclization and splitting contributes to the accumulation of lycopene in ripe tomato fruit.

In terms of reactive oxygen species, antioxidant enzymes are upregulated during chromoplast development, and lipids, rather than proteins, seem to be a target for oxidation. In the chromoplasts, an upregulation in the activity of superoxide dismutase and of components of the ascorbate-glutathione cycle was observed [31].

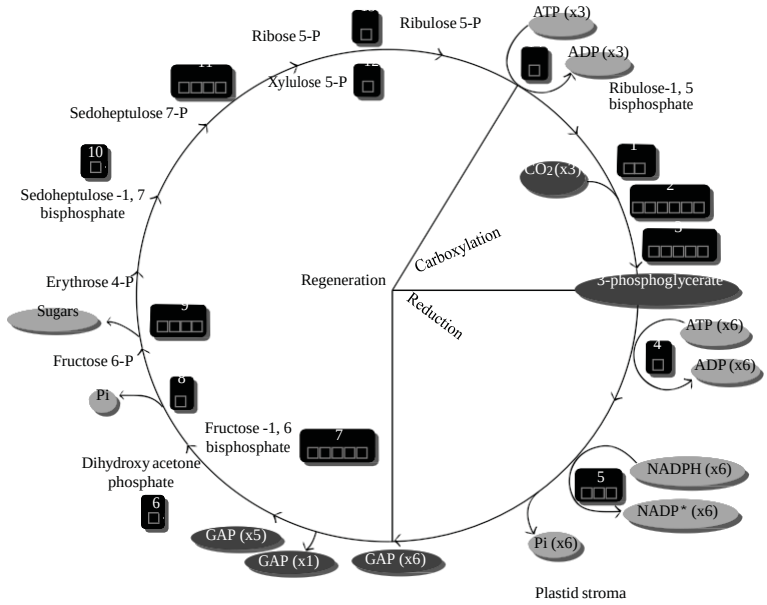
The plastid-to-nucleus signaling also undergoes important changes. In the chromoplast, the main proteins involved in the synthesis of Mg-protoporphyrin IX, a molecule supposed to play an important role in retrograde signaling [32] is absent, but other mechanisms such as hexokinase 1 or calcium signaling were present [16]. The plastid-nucleus communication is still an open subject with many still unanswered questions.

3. A Number of Metabolic Pathways Are Conserved during Chromoplast Differentiation

The comparison of data arising from proteomics of the chloroplast [33] and of the chromoplast [16] as well as biochemical analysis of enzyme activities suggest that several pathways are conserved during the transition from chloroplast to chromoplast. Such is the case for (i) the Calvin cycle which generates sugars from CO_2, (ii) the oxidative pentose phosphate pathway (OxPPP) which utilizes the 6 carbons of glucose to generate 5 carbon sugars and reducing equivalents, and (iii) many aspects of lipid metabolism (Figure 2). Activities of enzymes of the Calvin cycle have been measured in plastids isolated from sweet pepper. They may even be higher in chromoplasts than in chloroplasts [34] In ripening tomato fruits, several enzymes of the Calvin cycle (hexokinase, fructokinase, phosphoglucoisomerase, pyrophosphate-dependent phosphofructokinase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, and glucose 6-phosphate dehydrogenase) are active [35]. The activity of glucose 6-phosphate dehydrogenase (G6PDH), a key component of the OxPPP, was higher in fully ripe tomato fruit chromoplasts than in leaves or green fruits [36]. Also, a functional oxidative OxPPP has been encountered in isolated buttercup chromoplasts [37]. Proteomic analysis have demonstrated that an almost complete set of proteins involved in the OxPPP are present in isolated tomato fruit chromoplasts (Figure 2). The persistence of the Calvin cycle and the OxPPP cannot be related to photosynthesis since the photosynthetic system is disrupted. In nonphotosynthetic plastids, the Calvin cycle could provide reductants and also precursors of nucleotides and aromatic aminoacids to allow the OxPPP cycle to function optimally [16].

Starch transiently accumulates in young tomato fruit and undergoes almost complete degradation by maturity. In fact, starch accumulation results from an unbalance between synthesis and degradation. Enzymes capable of degrading starch have been detected in the plastids of tomato fruit. In addition, tomato fruit can synthesize starch during the period of net starch breakdown, illustrating that these two mechanisms can coexist [38]. As indicated in Figure 3, proteins for starch synthesis have been encountered in the tomato chromoplast (ADP-glucose pyrophosphorylase, starch synthase, and starch branching enzyme). In addition, the system for providing neutral sugars to the starch biosynthesis pathway is complete including the glucose-6P-translocator which imports sugars from the cytosol. The presence of active import of glucose-6P, but not glucose-1P, had been demonstrated in buttercup chromoplasts [37]. Although some starch granules may be present in ripe tomatoes, the amount of starch is strongly reduced [39]. The most probable explanation is that starch undergoes rapid turnover with intense degradation. This assumption is supported by the presence in the tomato chromoplast of most of the proteins involved in starch degradation (Figure 3). Particularly interesting is the presence of one glucan-water dikinase (GWD), one phospho-glucan-dikinase (PWD), and one phospho-glucan-phosphatase (PGP) that facilitate the action of β-amylases [40]. Mutants of these proteins, named starch excess (SEX1 corresponding to GWD and SEX4 to PGP), accumulate large amounts of starch [40]. In agreement with the above-mentioned hypothesis, high activity of β-amylase has been found during apple and pear fruit ripening at a time where starch has disappeared [41]. The presence of a glucose translocator for the export of sugars generated by starch degradation represents another support to the functionality of the starch metabolism pathways in chromoplasts. In olive fruit, a high expression of a glucose transporter gene was observed at full maturity when the chromoplasts were devoid of starch [42]. Nevertheless, the enzymatic activity of all of the proteins remains to be demonstrated inasmuch as posttranslational regulation of enzymes of starch metabolism has been reported [43] including protein phosphorylation [44]. Interestingly, orthologs of the 14-3-3 proteins of the μ family of Arabidopsis involved in the regulation of starch accumulation [45] are present in the tomato chromoplastic proteome (Figure 3). The 14-3-3 proteins participate in the phosphorylation-mediated regulatory functions in plants.

In chloroplasts, thylakoid membranes, as well as envelope membranes, are rich in galactolipids and sulfolipids [46]. Lipid metabolism is also highly active in the chromoplasts. Despite thylakoid disassembly, new membranes are synthesized such as those participating in the formation of

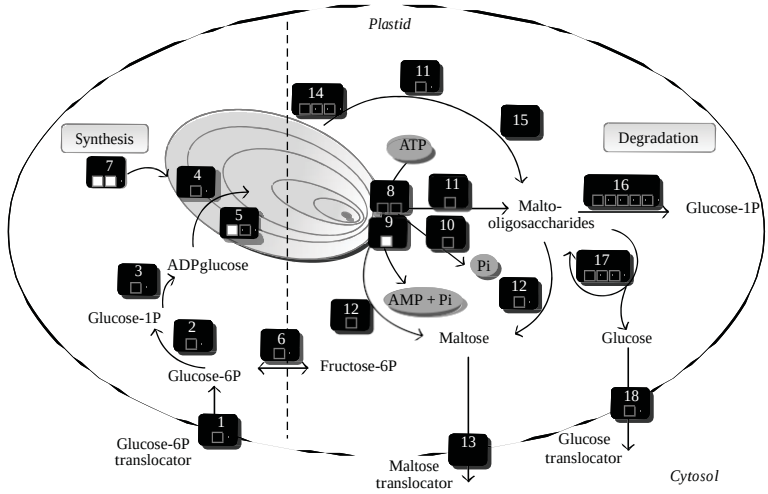


(a)

Carboxylation		
1	SGN-U346314	large subunit of RUBISCO
2	SGN-U314262	ribulose bisphosphate carboxylase small chain 1A
2	SGN-U314254	ribulose bisphosphate carboxylase small chain 1A
2	SGN-U314701	ribulose bisphosphate carboxylase small chain 3B
2	SGN-U314722	ribulose bisphosphate carboxylase small chain 3B
2	SGN-U314700	ribulose bisphosphate carboxylase small chain 3B
2	SGN-U338973	ribulose bisphosphate carboxylase small chain 3B
3	SGN-U316742	Chaperonin 60 beta
3	SGN-U312543	Rubisco activase
3	SGN-U312544	Rubisco activase
3	SGN-U312538	60 kDa chaperonin alpha subunit
3	SGN-U312542	60 kDa chaperonin alpha subunit
Reduction		
4	SGN-U313176	phosphoglycerate kinase
5	SGN-U312802	glyceraldehyde-3-phosphate dehydrogenase B subunit
5	SGN-U312804	glyceraldehyde-3-phosphate dehydrogenase B subunit
5	SGN-U312461	glyceraldehyde-3-phosphate dehydrogenase B subunit
Regeneration		
6	SGN-U313729	triose-phosphate isomerase
7	SGN-U314788	fructose-bisphosphate aldolase
7	SGN-U314787	fructose-bisphosphate aldolase
7	SGN-U312608	fructose-bisphosphate aldolase
7	SGN-U312609	fructose-bisphosphate aldolase
7	SGN-U312344	fructose-bisphosphate aldolase
8	SGN-U316424	fructose-1,6-bisphosphatase
9	SGN-U312320	Transketolase
9	SGN-U312319	Transketolase
9	SGN-U312322	Transketolase
9	SGN-U323721	Transketolase
10	SGN-U315559	sedoheptulose-bisphosphatase
11	SGN-U312320	Transketolase
11	SGN-U312319	Transketolase
11	SGN-U312322	Transketolase
11	SGN-U323721	Transketolase
12	SGN-U313308	ribulose-phosphate 3-epimerase
13	SGN-U315528	ribose 5-phosphate isomerase
14	SGN-U312791	phosphoribulokinase

(b)

Figure 2: Presence of proteins of the Calvin cycle in the tomato chromoplastic proteome. Proteins are indicated by white squares inside black frames and represented by their generic name and unigene SGN code. Numbers represent the position of the protein in the cycle. Data are derived from [16].



(a)

Starch synthesis		
1	SGN-U330538	Glucose-6-phosphate translocator
2	SGN-U324006	Phosphoglucomutase
2	SGN-U312467	Phosphoglucomutase
3	SGN-U317866	ADP-glucosepyro phosphorylase
4	SGN-U318293	Starch synthase I
5	SGN-U312423	Starch branching enzyme
5	SGN-U312427	Starch branching enzyme
6	SGN-U317897	Phosphoglucose isomerase
Post translational regulation of starch synthesis		
7	SGN-U313499	14-3-3-like protein GF14 mu
7	SGN-U316857	14-3-3-like protein GF14 mu
Starch degradation		
8	SGN-U328612	Phosphoglucan-water dikinase
9	SGN-U315116	Glucan-water dikinase or SEX1
10	SGN-U317732	Phosphoglucan phosphatase or SEX4
11	SGN-U328875	Isoamylase3
11	SGN-U333011	Isoamylase3
12	SGN-U313315	β amylase3
13	ABSENT	Maltose translocator or RCP1
14	SGN-U317456	α amylase3
14	SGN-U326232	α amylase3
14	SGN-U326817	α amylase3
15	ABSENT	Limit dextrinase
16	SGN-U316416	α glucan phosphorylase
16	SGN-U316417	α glucan phosphorylase
16	SGN-U333374	α glucan phosphorylase
16	SGN-U325849	α glucan phosphorylase
16	SGN-U345057	α glucan phosphorylase
17	SGN-U322816	Disproportionating enzyme 1
17	SGN-U333138	Disproportionating enzyme 1
17	SGN-U342143	Disproportionating enzyme 1
18	SGN-U319050	Glucose translocator

(b)

Figure 3: Presence of proteins of the starch synthesis and degradation pathways, of posttranslational regulation of starch synthesis, and of sugar translocators in the tomato chromoplastic proteome. Proteins are indicated by white squares inside black frames and represented by their generic name and unigene SGN code. Numbers represent the position of the protein in the cycle. Data are derived from [16].

carotenoid storage structures. These newly synthesized membranes are not derived from the thylakoids but rather from vesicles generated from the inner membrane of the plastid [47]. Key proteins for the synthesis of phospholipids, glycolipids, and sterols were identified [16] along with some proteins involved in the lipoxygenase (LOX) pathway. They have been described in the chloroplast, and they lead to the formation oxylipins, which are important compounds for plant defense responses [48]. In the tomato chromoplast, all proteins potentially involved in the LOX pathway leading to the generation of aroma volatiles were found [16].

The shikimate pathway, which is present in microorganisms and plants and never in animals, is a branch point between the metabolism of carbohydrates and aromatic compounds. It leads to the biosynthetic of the aromatic amino acids tyrosine, tryptophan, and phenylalanine [49]. The presence of an active shikimate pathway has been demonstrated in chromoplasts isolated from wild buttercup petals by measuring the activity of the shikimate oxidoreductase [50], and a number of proteins involved in the shikimate pathway have been encountered in the tomato chromoplast proteome [16]. The aromatic amino acids derived from the shikimate pathway are the precursors of a number of important secondary metabolites. Tyrosine is the precursor of tocopherols and tocotrienols. Tryptophane is involved in the synthesis of indole alkaloids which are essential for the generation of some glucosinolates, terpenoids, and tryptamine derivatives [50]. Phenylalanine is the precursor of several classes of flavonoids, including anthocyanins. It is also a precursor for the biosynthesis of volatile compounds which are important for fruit flavor and flower scent, eugenol, 2-phenylacetaldehyde and, 2-phenylethanol [51, 52]. In tomato fruit, for instance, 2-phenylacetaldehyde and 2-phenylethanol are generated from phenylalanine by an aromatic amino acid decarboxylase and a phenylacetaldehyde reductase, respectively [53, 54]. Nevertheless, there is no indication that the synthesis of the secondary metabolites derived from the shikimate pathway takes place in the chromoplast.

During fruit ripening, an increased synthesis of α-tocopherol was observed [55]. The biosynthesis of α-tocopherol was localized in the envelope membranes of the *Capsicum annum* [56], and the almost complete set of proteins of the pathway were present in the tomato chromoplast [16]. The accumulation of α-tocopherol, by protecting membrane lipids against oxidation, may contribute to delaying senescence [57].

4. Plastoglobuli, Plastoglobules, and the Chloroplast-to-Chromoplast Transition

Plastoglobules are lipoprotein particles present in chloroplasts (Figure 1) and other plastids. They have been recently recognized as participating in some metabolic pathways [58]. For instance, plastoglobules accumulate tocopherols and harbor a tocopherol cyclase, an enzyme catalyzing the conversion of 2,3-dimethyl-5-phytyl-1,4-hydroquinol to γ-tocopherol [59]. Plastoglobuli also accumulate carotenoids as crystals or as long tubules named fibrils [60, 61]. Part of the enzymes involved in the carotenoid biosynthesis pathway (ζ-carotene desaturase, lycopene β cyclase, and two β-carotene β hydroxylases) were found in the plastoglobuli [62].

Plastoglobules arise from a blistering of the stroma-side leaflet of the thylakoid membrane [63], and they are physically attached to it [45]. During the chloroplast-to-chromoplast transition, a change in the size and number of plastoglobuli was observed (Figure 1). They are larger and more numerous than in the chloroplast [7]. Plastoglobules are the predominant proteins of plastoglobules. Several types of plastoglobules have been described: fibrillin, plastid lipid-associated proteins (PAP) and carotenoid-associated protein (CHRC). All plastoglobules participate in the accumulation of carotenoids in the plastoglobule structure. Carotenoids accumulate as fibrils to form supramolecular lipoprotein structures. A model for fibril assembly has been proposed in which the core is occupied by carotenoids that interact with polar galacto- and phospho-lipids. Fibrillin molecules are located at the periphery in contact with the plastid stroma [64]. In tomato, the overexpression of a pepper fibrillin caused an increase in carotenoid and carotenoid-derived flavour volatiles [47] along with a delayed loss of thylakoids during the chloroplast-to-chromoplast transition. In fibrillin overexpressing tomato, the plastids displayed a typical chromoplastic zone contiguous with a preserved chloroplastic zone. PAP is another major protein of plastoglobules that also participates in the sequestration of carotenoids [64, 65]. As for CHRC, its downregulation resulted in a 30% reduction of carotenoids in tomato flowers [66]. Plastoglobuli are, therefore, complex assemblies that play a key role in carotenoid metabolism and greatly influence the evolution of the internal structure of the plastid during the chloroplast to chromoplast transition.

5. A key Player in Chromoplast Differentiation: The Or Gene

The *Or* gene was discovered in cauliflower where the dominant mutation *Or* conferred an orange pigmentation with the accumulation of β-carotene mostly in the inflorescence [67]. The *Or* gene was isolated by positional cloning [68]. It is localized in the nuclear genome and is highly conserved among divergent plant species [69]. The *Or* protein corresponds to plastid-targeted a DnaJ-like co-chaperone with a cysteine-rich domain lacking the J-domain [68]. DnaJ proteins are known for interacting with Hsp70 chaperones to perform protein folding, assembly, disassembly, and translocation. The *Or* mutation is not a loss of function mutation as indicated by the absence of phenotype upon RNAi silencing. It is probably a dominant-negative mutation affecting the interaction with Hsp70 chaperones [70]. The *OR* mutants displayed an arrest in plastid division so that a limited number of chromoplasts (one or two) were present in the affected cells [71]. Potato tubers over-expressing the *Or* gene accumulate carotenoids [69]. In the *OR* mutant, the expression of carotenoid biosynthetic genes was unaffected

and chromoplasts differentiated normally with membranous inclusions of carotenoids similar to those of carrot roots. It is concluded that the *Or* gene is not involved in carotenoid biosynthesis but rather creates a metabolic sink for carotenoid accumulation through inducing the formation of chromoplasts [72].

6. Transcriptional and Translational Activity in the Plastid Undergo Subtle Changes during Chromoplast Biogenesis

Most proteins present in the plastid are encoded by nuclear genes. The plastid genome encodes around 84 proteins [60]. Restriction enzyme analysis between chloroplasts of leaves and chromoplasts of tomato fruit indicates the absence of rearrangements, losses, or gains in the chromoplastic DNA [61]. During chromoplast differentiation, the global transcriptional activity is stable, except for a limited number of genes such as *accD*, encoding a subunit of the acetyl-CoA carboxylase involved in fatty acid biosynthesis, *trnA* (tRNA-ALA), and *rpoC2* (RNA polymerase subunit) [15]. Polysome formation within the plastids declined during ripening suggesting that, while the overall RNA levels remain largely constant, plastid translation is gradually downregulated during chloroplast-to-chromoplast differentiation. This trend was particularly pronounced for the photosynthesis gene group. A single exception was observed; the translation of *accD* stayed high and even increased at the onset of ripening [15].

Specific studies of few plastid-localized genes have been carried out. Genes involved in photosynthesis were, as expected, downregulated during chromoplast formation [25]. However, an upregulation of the large subunit of *ribulose-1,5-bisphosphate carboxylase/oxygenase* and the 32 kD *photosystem II quinone binding protein* genes has been observed in the chromoplasts of squash fruits (*Cucurbitae pepo*) [62]. A possible explanation would be that these genes could be regulated independently from the plastid differentiation processes. Genes involved in carotenoid biosynthesis such as the *lycopene β-cyclase* (*CYCB*) were upregulated during chromoplast formation in many plants including the wild species of tomato *Solanum habrochaites* [63].

7. Changes in Gene Expression during Chromoplast Differentiation in Ripening Tomato

The availability of proteomic data of tomato chromoplasts [16] and expression data of a wide range of tomato genes (The Tomato Expression Database: http://ted.bti.cornell.edu) [73] allowed classifying genes encoding chromoplastic proteins according to their expression pattern (Table 1). Among the 87 unigenes whose encoded proteins are located in the chromoplast, the biggest functional class corresponds to genes involved in photosynthesis. Most of them (18 out of 24) are either permanently (Table 1(c)) or transiently (Table 1(e)) downregulated at the breaker stage. This is in agreement with the dramatic decrease in the photosynthetic activity of the chromoplast. Three of them show constant expression (Table 1(a): U313693 ATP synthase delta chain; U312985 glycine cleavage system H protein; U312532 oxygen-evolving enhancer protein) and three upregulation (Table 1(b): U312690 plastocyanin; U312593 chlorophyll A-B binding protein 4; U314994 phosphoglycolate phosphatase). In the case of Calvin cycle, 5 out of 12 genes (U312344 fructose-bisphosphate aldolase; U312608 fructose-bisphosphate aldolase; U312609 fructose-bisphosphate aldolase; U314254 ribulose bisphosphate carboxylase small chain 1A; U314701 ribulose bisphosphate carboxylase small chain 3B) had a constant decrease during chromoplast differentiation (Table 1(c)). In tomato fruit, the activity of the ribulose-1,5-bisphosphate carboxylase/oxygenase had a constant decrease during fruit ripening [74], which is in line with the transcriptomic and proteomic data. The genes encoding fructose-bisphosphate aldolase isoforms presented different expression profiles being either up- (U314788) or down- (U312344) regulated during tomato fruit ripening. An increase in overall transcript levels for the fructose-1,6-bisphosphate aldolase has been described during ripening [75]. The importance of transcripts and enzyme activity of the various isoforms are unknown. The remaining genes involved in the Calvin cycle showed either increased (Table 1(b); U312802 glyceraldehyde-3-phosphate dehydrogenase B; U312538 RuBisCO subunit binding-protein) or unchanged expression (Table 1(a); U316424 fructose-1,6-bisphosphatase; U312544 ribulose bisphosphate carboxylase/-oxygenase activase). Three genes coding for the OxPPP were found: two of them exhibited a transient increase in expression at the breaker stage (Table 1(d): U315528 ribose 5-phosphate isomerase-related; U332994 6-phosphogluconate dehydrogenase family protein) and one a transient decrease (Table 1(e): U315064 transaldolase). The 3 genes involved in tetrapyrrole biosynthesis are not part of the chlorophyll synthesis branch and all of them had an increased expression (Table 1(b): U315993 coproporphyrinogen III oxidase; U315267 uroporphyrinogen decarboxylase; U315567 hydroxymethylbilane synthase), suggesting that the synthesis of tetrapyrroles continues during the transition from chloroplast to chromoplast. As expected, most of the genes (5 out of 6) coding for enzymes involved in carotenoid synthesis showed continuous (Table 1(b): U314429 phytoene synthase; U315069 isopentenyl-diphosphate delta-isomerase II; U316915 geranylgeranyl pyrophosphate synthase; U318137 phytoene dehydrogenase) or transient (Table 1(d): U313450 geranylgeranyl reductase) upregulation. The precursors for carotenoid production are synthesized through the methylerythritol phosphate (MEP) pathway. The gene encoding hydroxymethylbutenyl 4-diphosphate synthase (HDS) (U314139) downstream in the pathway has stable expression (Table 1(a)). This is consistent with previous studies that showed that there were no significant changes in *HDS* gene expression during tomato fruit ripening [76].

Table 1: Expression profile analysis of 87 genes whose products are targeted to tomato chromoplasts (*).

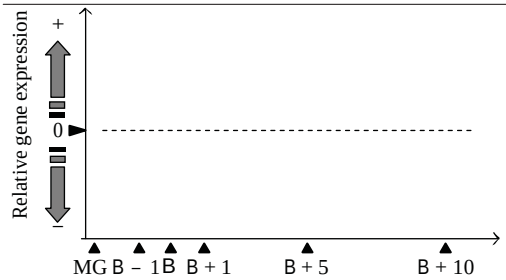
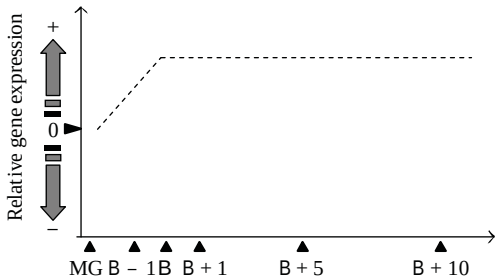
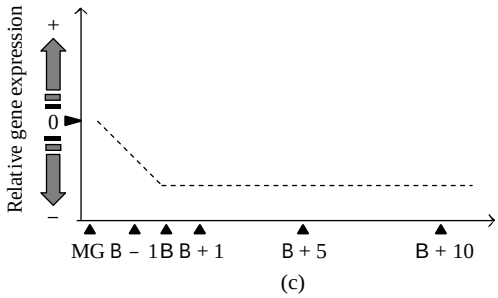
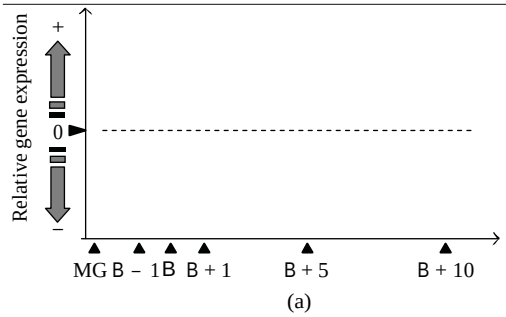
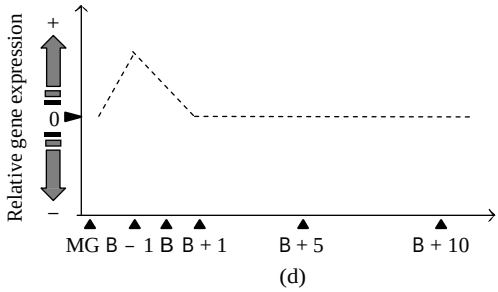
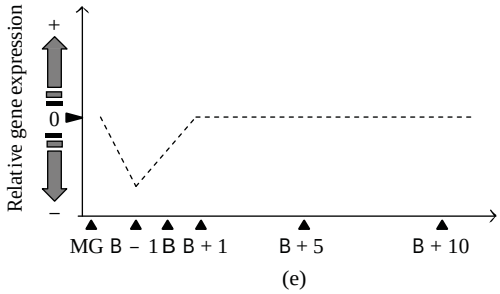
 (a)	*Photosystem*: U313693 ATP synthase delta chain; U312985 glycine cleavage system H protein; U312532 oxygen-evolving enhancer protein. *Calvin cycle*: U316424 fructose-1, 6-bisphosphatase; U312544 ribulose bisphosphate carboxylase/-oxygenase activase. *Secondary metabolism*: U314139 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase.
 (b)	*Photosystem*: U312690 plastocyanin; U312593 chlorophyll A-B binding protein 4; U314994 phosphoglycolate phosphatase. *Calvin cycle*: U314788 fructose-bisphosphate aldolase; U312802 glyceraldehyde-3-phosphate dehydrogenase B; U312538 RuBisCO subunit binding-protein. *Redox*: U314092 L-ascorbate peroxidase; U319145 thioredoxin family protein; U320487 monodehydroascorbate reductase. *Amino acid metabolism*: U321505 anthranilate synthase; U317466 3-phosphoshikimate 1-Carboxyvinyltransferase; U317564 tryptophan synthase. *Lipid metabolism*: U315474 3-oxoacyl-(acyl-carrier-protein) synthase I; U315475 3-oxoacyl-(acyl-carrier-protein) synthase I; U313753 pyruvate dehydrogenase E1 component. Major CHO metabolism: U315116 starch excess protein (SEX1); U333011 isoamylase, putative; U312423 1, 4-alpha-glucan branching enzyme; U312427 1, 4-alpha-glucan branching enzyme. *Secondary metabolism*: U314429 phytoene synthase; U315069 isopentenyl-diphosphate delta-isomerase II; U316915 geranylgeranyl pyrophosphate synthase; U318137 phytoene dehydrogenase. *Tetrapyrrole synthesis*: U315993 coproporphyrinogen III oxidase; U315267 uroporphyrinogen decarboxylase; U315567 hydroxymethylbilane synthase. *Mitochondrial electron transport*: U316255 NADH-ubiquinone oxidoreductase. *Fermentation, ADH*: U314358 alcohol dehydrogenase (ADH). *Miscellaneous, cytochrome P450*: U313813 NADPH-cytochrome p450 reductase. *S-assimilation. APS*: U313496 sulfate adenylyltransferase 1. *Development unspecified*: U316277 senescence-associated protein (SEN1). *Cell organisation:* U313480 plastid lipid-associated protein PAP, putative. *Hormone metabolism*: U315633 lipoxygenase. *N-metabolism ammonia metabolism*: U323261 glutamate synthase (GLU1). *Stress abiotic heat*: U315717 HS protein 70. *Not assigned, No ontology*: U317890 hydrolase, alpha/beta fold family protein.
 (c)	*Photosystem*: U312531 oxygen-evolving enhancer protein; U313447 photosystem I reaction center subunit IV; U313204 chlorophyll A-B binding protein 2; U313245 ATP synthase gamma chain 1; U312436 chlorophyll A-B binding protein; U313211 chlorophyll A-B binding protein 2; U313212 chlorophyll A-B binding protein 2; U313213 chlorophyll A-B binding protein 2; U312572 photosystem II oxygen-evolving complex 23 (OEC23); U314260 photosystem I reaction center subunit III family protein. *Calvin cycle*: U312344 fructose-bisphosphate aldolase; U312608 fructose-bisphosphate aldolase; U312609 fructose-bisphosphate aldolase; U314254 ribulose bisphosphate carboxylase small chain 1A; U314701 ribulose bisphosphate carboxylase small chain 3B. *Lipid metabolism*: U319207 phosphatidylglycerol phosphate synthase (PGS1). *Redox*: U313537 dehydroascorbate reductase. *Major CHO metabolism*: U316416 glucan phosphorylase, putative. *N-metabolism*: U314517 glutamine synthetase (GS2). *Amino acid metabolism*: U317344 bifunctional aspartate kinase/homoserine dehydrogenase; U320667 cystathionine beta-lyase.

Table 1: Continued.

 (a)	*Photosystem*: U313693 ATP synthase delta chain; U312985 glycine cleavage system H protein; U312532 oxygen-evolving enhancer protein. *Calvin cycle*: U316424 fructose-1, 6-bisphosphatase; U312544 ribulose bisphosphate carboxylase/-oxygenase activase. *Secondary metabolism*: U314139 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase.
 (d)	*Redox*: U314061 peroxiredoxin Q; U314093 L-ascorbate peroxidase, thylakoid-bound (tAPX); U314923 2-cys peroxiredoxin. *OPP, Nonreductive PP*: U315528 ribose 5-phosphate isomerase related; U332994 6-phosphogluconate dehydrogenase family protein; U316131 6-phosphogluconate dehydrogenase NAD-binding domain-containing protein. *Secondary metabolism*: U317741 acetyl coenzyme A carboxylase carboxyl transferase alpha subunit family; U313450 geranylgeranyl reductase. *Amino acid metabolism*: U317245 tryptophan synthase related. *N-metabolism ammonia metabolism*: U317524 ferredoxin-nitrite reductase. *Lipid metabolism*: U315697 enoyl-(acyl-carrier protein) reductase (NADH) U321151 lipoxygenase. *Transport metabolite*: U312460 triose phosphate/phosphate translocator, putative. *Signalling calcium*: U315961 calnexin 1 (CNX1).
 (e)	*Photosystem*: U312843 chlorophyll A-B binding protein; U312858 cytochrome B6-F complex iron-sulfur subunit; U313214 chlorophyll A-B binding protein 2; U312791 phosphoribulokinase (PRK); U317040 photosystem II reaction center PsbP family protein; U312449 chlorophyll A-B binding protein CP26; U312661 chlorophyll A-B binding protein CP29 (LHCB4); U313789 ATP synthase family. *Calvin cycle*: U312461 glyceraldehyde 3-phosphate dehydrogenase A; U312871 oxygen-evolving enhancer protein 3. *Redox*: U315728 glutathione peroxidase. *OPP nonreductive PP transaldolase*: U315064 transaldolase. *Signaling calcium*: U318939 calcium-binding EF hand family protein. *Major CHO metabolism*: U313315 beta amylase.

(*) Genes represented in this table are filtered from TED database [64] crossing with the proteins described by Barsan et al. [16]. The expression profiles were clustered with the Bioinformatics tools of the Matlab (MathWorks) software package and further reduced to five representative expression profiles according to their general tendencies represented in the first column. The expression values used in this analysis were taken from experiment E011 from TED database. Relative expression refers to the ratio between the expression values of each ripening point and MG. All data were normalized by the mean and log2 transformed. (a) Genes that remain stable during the ripening, (b) genes that have an increase or (c) a decrease until breaker stage and then reaches a plateau, (d) genes that have a positive or (e) negative transient expression around the breaker stage. (MG, mature green; *B*-1, 1 d before breaker; *B*, breaker stage; *B* + 1, 1 d after breaker; *B* + 5, 5 d after breaker; *B* + 10, 10 d after breaker.)

In the case of lipid metabolism, three genes showed increased expression (Table 1(b): U315474 3-oxoacyl-(acyl-carrier-protein) synthase I; U315475 3-oxoacyl-(acyl-carrier-protein) synthase I; U313753 pyruvate dehydrogenase E1 component), and two genes had transient increase (Table 1(d): U315697 enoyl-(acyl-carrier protein) reductase (NADH); U321151 lipoxygenase). Phosphatidylglycerol phosphate synthase showed decreased expression (Table 1(c)). This enzyme is involved in the biosynthesis of phosphatidylglycerol and is considered as playing an important role in the ordered assembly and structural maintenance of the photosynthetic apparatus in thylakoid membranes and in the functioning of the photosystem II [77]. The downregulation of this gene during chromoplast differentiation is consistent with thylakoid disintegration and photosynthesis disappearance.

Four genes of the starch metabolism present upregulation (Table 1(b)). Two of them are part of the starch biosynthesis (U312423 1,4-alpha-glucan branching enzyme; U312427 1, 4-alpha-glucan branching enzyme), and one of them is involved starch degradation U315116 starch excess protein (SEX1). The fourth one, an isoamylase (U333011) can participate either in starch degradation or in starch synthesis, depending on the isoform [78]. The expression of the gene that codes a starch degrading glucan phosphorylase (U316416) decreases, and the expression of another starch degrading gene, beta-amylase (U313315), has a negative transient expression (Table 1(b)). In addition, proteomic

studies have shown the presence of two starch excess proteins (SEX1 and 4) that probably contribute to the absence of starch accumulation [16]. Starch is degraded during the chloroplast to chromoplast transition to provide carbon and energy necessary to sustain the metabolic activity during fruit ripening. Several enzymes are responsible for the processes, each one possessing several isoforms with different regulatory mechanisms [78].

Interestingly genes involved in aroma production such as *ADH* (U314358) or *LOXC* (U315633) had a constant increase in gene expression (Table 1(b)). This could be related to the increase in aroma production via the LOX pathway.

The microarray data discussed in this section cover a wide range of the tomato transcriptome. However, several isoforms of several genes are not represented in the database, which could explain some contradictory patterns of expression encountered in our analysis. Nevertheless, although not providing a full picture of the molecular events occuring during the chloroplast to chromoplast transition, these data confirm the regulation at the transcriptional level of the most salient events.

8. Conclusions and Perspectives

With the advent of high throughput technologies, great progress has been made in the recent years in the elucidation of the structure and function of plastids. The most important data obtained in the area have been generated for the chloroplast of Arabidopsis. Much less information is available for the chromoplast. However, recent studies with bell pepper [14] and tomato fruit [16] have allowed assigning to chromoplasts a number of proteins around 1000, which is in the same order of magnitude as Arabidopsis chloroplasts [33]. This number is, however, much lower than the number of proteins predicted to be located in the plastid which has been estimated at up to 2700 [79] or even 3800 [80]. The increased sensitivity of the mass spectrometry technologies associated with efficient methods of purification of plastids, particularly chromoplasts, will allow in the future identifying more proteins. So far, changes in the proteome have not been described during the differentiation of chromoplast. Such studies imply the development of efficient protocols for isolating plastids at different stages of differentiation during chromoplastogen- esis. The combination of proteomics and transcriptomics may also give novel information on the process in a near future. The discovery of the *Or* gene has been a great step forward to the understanding of the molecular deter- minism of chromoplast differentiation. There is a need to better understand the regulatory mechanism controlling the expression of the *Or* gene. Many genes encoding for plastidial proteins are regulated by the plant hormone ethylene and, therefore, participate in the transcriptional regulation of the fruit ripening process in general [81, 82]. Other hormones such as ABA and auxin may also be involved. Interactions between hormones and other signals (light, for instance) during chromoplast differentiation represent another field of investigation to be explored. Because most of the proteins present in the chromoplast are encoded by nuclear genes, it will be important in future to better understand the changes occurring in the processes of transport of proteins to the chromoplast. It is suspected that vesicular transport is gaining importance, but more experimental evidence is required. Finally the dialog between the nucleus and the chromoplast and the signals involved needs to be explored. So far most of the studies in this area have been carried out with chloroplasts [83].

In conclusion, important steps forward have been made into a better understanding of chromoplast differentiation. Metabolic reorientations and specific biochemical and molecular events have been clearly identified. It is predictable that more information will arise from the indepth description of the molecular events occurring during the chloroplast to chromoplast transition using genomic tools.

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Chapter I-Chloroplast to chromoplast transition in tomato fruit: spectral confocal microscopy analyses of carotenoids and chlorophylls in isolated plastids and time-lapse recording on intact live tissue

Introduction

Tomato fruit development is a process associated with lots of changes, such as fruits size expansion, aroma accumulation and color change. All these changes help the seed dispersal of fleshy fruits (Pyke, 2008). The color change from green to red is one of the most obvious feature during fruit development. Pigments contributing to the fruit color from green to red are chlorophyll and carotenoid, respectively. Pyke (2007) showed a strong evidence that chromoplast is derived from chloroplast using green fluorescent protein (GFP). But there no observation of carotenoid fluorescence was performed.

In the present work, we observed the pigment fluorescence within individual plastids with a laser scanning confocal microscope. The plastids were isolated from tomato fruits at three different ripening stages. Then we performed a real-time monitoring of these color changes within live tomato fruit slices. The observation of individual plastid and real-time monitoring of the transition revealed that all the chromoplasts derived from pre-existing chloroplasts in tomato tissues.

This work was published in Annals of Botany for which I participated as the first co-author.

Foreword:

For the work presented in this chapter I participated in isolation of the plastid from three different stages (mature-green, breaker and red), the assessment of the intactness of isolated plastids, the observation of isolated plastids by confocal microscopy and assay of the fluorescence emission spectra.

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Chloroplast to chromoplast transition in tomato fruit: spectral confocal microscopy analyses of carotenoids and chlorophylls in isolated plastids and time-lapse recording on intact live tissue

Isabel Egea[1,2,†], Wanping Bian[1,2,4,†], Cristina Barsan[1,2], Alain Jauneau[3], Jean-Claude Pech[1,2], Alain Latché[1,2], Zhengguo Li[4,*] and Christian Chervin[1,2,*]

[1]Université de Toulouse, INP-ENSA Toulouse, Génomique et Biotechnologie des Fruits, Avenue de l'Agrobiopole, BP 32607, Castanet-Tolosan, F-31326, France, [2]INRA, Génomique et Biotechnologie des Fruits, Chemin de Borde Rouge, Castanet-Tolosan, F-31326, France, [3]Université de Toulouse, CNRS, IFR40, Pôle de Biotechnologie Végétale, Chemin de Borde Rouge, Castanet-Tolosan, F-31326, France and [4]Genetic Engineering Research Centre, Bioengineering College, Chongqing University, Chongqing 400044, PR China

* For correspondence. E-mail chervin@ensat.fr or zhengguoli@cqu.edu.cn

[†]These authors contributed equally to this work.

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† Background and Aims There are several studies suggesting that tomato (Solanum lycopersicum) chromoplasts arise from chloroplasts, but there is still no report showing the fluorescence of both chlorophylls and carotenoids in an intermediate plastid, and no video showing this transition phase.

† Methods Pigment fluorescence within individual plastids, isolated from tomato fruit using sucrose gradients, was observed at different ripening stages, and an in situ real-time recording of pigment fluorescence was performed on live tomato fruit slices.

† Key results At the mature green and red stages, homogenous fractions of chloroplasts and chromoplasts were obtained, respectively. At the breaker stage, spectral confocal microscopy showed that intermediate plastids contained both chlorophylls and carotenoids. Furthermore, an in situ real-time recording (a) showed that the chloroplast to chromoplast transition was synchronous for all plastids of a single cell; and (b) confirmed that all chromoplasts derived from pre-existing chloroplasts.

† Conclusions These results give details of the early steps of tomato chromoplast biogenesis from chloroplasts, with the formation of intermediate plastids containing both carotenoids and chlorophylls. They provide information at the sub-cellular level on the synchronism of plastid transition and pigment changes.

Key words: Chloroplast, chromoplast, confocal, pigment fluorescence, Solanum lycopersicum, tomato.

INTRODUCTION

During evolution, chromoplasts have emerged as plastid structures which accumulate pigments to facilitate flower pollination and seed dispersal of fleshy fruit. There is good evidence that chromoplasts derive from chloroplasts (Pyke, 2007), even if nobody has ever recorded this transition. Structural changes occurring during chloroplast to chromoplast transition have been described in fleshy fruit by electron microscopy primarily in tomato (Rosso, 1968; Harris and Spurr, 1969) and in bell pepper (Spurr and Harris, 1968). During the differentiation process controlled breakdown of chlorophyll and disruption of the thylakoid membrane occurred, concomitant with an increase in the aggregation of carotenoids. Different carotenoid-accumulating bodies have been described, including plastoglobules, crystalline and microfibrillar structures, and internal membranous structures (Marano et al., 1993). Coloured images have been made under bright-field microscopy of plastids of fruit and flowers where chloroplasts appear as dark green pigmented bodies, while chromoplasts appear as dark red or orange bodies (Gunning, 2005). More recently, Pyke and co-workers (Pyke and Howells, 2002; Waters et al., 2004; Forth and Pyke, 2006; Pyke, 2007) have studied chloroplast to chromoplast transition using green fluorescent protein (GFP) constructs targeted to the plastid by the RecA plastid transit sequence. This technique showed the highly irregular and variable morphology of plastids between different types of cells. It also allowed the morphology of stromules to be studied and the presence of bead-like structures along the stromules to be discovered. In Pyke and Howells (2002), the fluorescence of carotenoids might have been quenched by the use of an anti-fading compound, Vectashield[w] (http://forums.biotechniques.com/viewtopic.php?f=17&t=22802); therefore, chromoplasts were observed in ripe fruit tissues using the merging of GFP fluorescence and bright-field images, but the transition from chloroplasts was not observed. In later studies (Waters et al., 2004; Forth and Pyke, 2006), the authors focused on chlorophyll and GFP fluorescence during the transition from chloroplasts to chromoplasts and then onto stromules. No observation with carotenoid fluorescence was performed. In the present work, a laser scanning confocal microscopy technique has been

used to monitor the loss of chlorophylls and the accumulation of carotenoids simultaneously in individual plastids isolated at different stages of development. Time-lapse recording on tissue slices of tomato fruit was also performed to follow the transition in situ in a cellular set of plastids.

MATERIALS AND METHODS

Plant material

Tomato plants (Solanum lycopersicum 'MicroTom') were germinated and cultivated under greenhouse conditions and fruit was collected at the mature green, turning (2 d after breaker, Br + 2) and ripe (10 d after breaker, Br + 10) stages as shown in Egea et al. (2010). The breaker stage is characterized by the initiation of fruit colouration, with fruit changing from green to pale orange at the blossom end.

Plastid isolation and intactness assessment

Fruit were thoroughly washed with distilled water, the seeds and gel were eliminated and the pericarp was cut into small pieces (0·5–1·0 cm). Prior to homogenization, small fruit pieces were incubated in ice-cold extraction buffer (250 mM HEPES, 330 mM sorbitol, 0·5 mM EDTA, 5 mM b-mercaptoethanol, pH 7·6) for 30 min. Intact purified plastids were obtained by differential and density gradient centrifugation in discontinuous gradients of sucrose, as previously described by Barsan et al. (2010) with some modifications. Fruit for extraction of chloroplasts, immature chromoplasts and mature chromoplasts were chosen from mature green, breaker and ripe fruit, respectively, as described above. For isolating chloroplasts, a three-layer sucrose gradient was used, 0·9 M–1·15 M–1·45 M, and for mature chromoplasts the sucrose gradients were 0·5 M–0·9 M–1·35 M. To obtain intact purified immature chromoplasts, from breaker fruit, a more sensitive discontinuous sucrose density gradient was necessary (0·5 M–0·9 M–1·15 M–1·25 M–1·35 M–1·45 M). Intact chloroplasts, immature chromoplasts and mature chromoplasts banded in the 1·15 M–1·45 M, 0·9 M–1·15 M and 0·9 M–1·35 M sucrose interfaces, respectively. The plastid bands collected were washed twice with extraction buffer and finally resuspended in extraction buffer for further confocal microscopy observations.

For the intactness assessment, the isolated plastids were suspended in a buffer containing 25 mM Bicine, 25 mM HEPES, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0·4 M sorbitol, pH 9 supplemented with an equal volume of carboxyfluorescein diacetate (CFDA) at a final concentration of 0·0025 % (w/v) and incubated for 5 min (Schulz et al., 2004). CFDA fluoresces strongly when it is de-esterified to carboxyfluorescein in an intact plastid. Plastid suspensions were examined using an inverted microscope (Leica DMIRBE) equipped with an I3 cube filter (excitation filter 450–490 nm, dichroic mirror 510 nm and emission filter LP 515 nm). The number of total plastids per microlitre in the samples was determined using a haemacytometer (Neubauer Double, Zuzi), and the results were expressed as a percentage of intact plastids.

Tomato mesocarp preparation for in situ time-lapse recording

Very thin slices (around 300 mm) of tomato mesocarp were hand cut with a razor blade on the green part of inner + outer mesocarp layers, at the turning stage (Br + 2). Slices were mounted on a glass slide in water (as shown in Supplemenatary Data Fig. S1A, available online), containing 0·1 mM of the ethylene precursor 1-aminocyclopropane-1-car boxylic acid (ACC). This treatment enhanced the chance of observing colour changes within a time interval (several hours), during which the tissues neither moved on the slide nor dehydrated.

Confocal microscopy of isolated plastids

Confocal images of isolated plastids from the different fractions were acquired with a laser scanning confocal system (Leica LSCM-SP2, Nanterre, France) coupled with an upright microscope (Leica DM6000, Rueil-Malmaison, France). Samples of freshly isolated plastid fractions were placed between a glass slide and a coverslip. Fluorescence emission spectra were acquired using the 488 nm ray line of an argon laser for excitation and the emitted fluorescence was recorded from 505 to 745 nm with a bandwidth of 10 nm using the **l**-scan module of the Leica software. The fluorescence intensity expressed with arbitrary units corresponds to the average intensity of fluorescence per pixel.

Confocal microscopy for time-lapse recording on mesocarp tissues

Time-lapse acquisitions were performed using a long working distance ×40 water immersion lens (NA: 0·8) dipping directly in the buffer. Slices of the mesocarp tissue were placed at the bottom of a Petri dish filled with buffer to avoid both displacement and dehydration during the time-lapse acquisition (shown in Supplementary Data Fig. S1B). A hole in the cover of the Petri dish allowed the objective lens to dip in the buffer. The whole apparatus (Petri dish and objective lens) was covered with Parafilm^{w} to avoid dehydration. The 488 nm excitation ray line of an argon laser was used to image the distribution of carotenoids, the emitted fluorescence being collected between 500 and 600 nm. For chlorophylls, the 633 nm excitation ray line of a He:Ne laser was used and the emitted fluorescence between 650 and 700 nm was collected. Over 18·5 h, images were acquired every 15 min taking advantage of the time-lapse module of the Leica software. To reduce photodamage due to laser illumination, both laser intensities were minimized, and image averaging was not performed. To collect fluorescence emission, the pinhole was opened at Airy ¼ 2 and the photomultiplier gain increased, which generated some electronic noise, especially for the carotenoid channel. Overall, the photobleaching was reduced during image acquisition. Measurements of fluorescence intensity were performed on the raw t-series with Image-Pro software. There was more variability in the carotenoid signal than in the chlorophyll signal. This may be due to a differential setting of the photomultiplier in the two different channels. As an increase in carotenoid signal was expected, to avoid

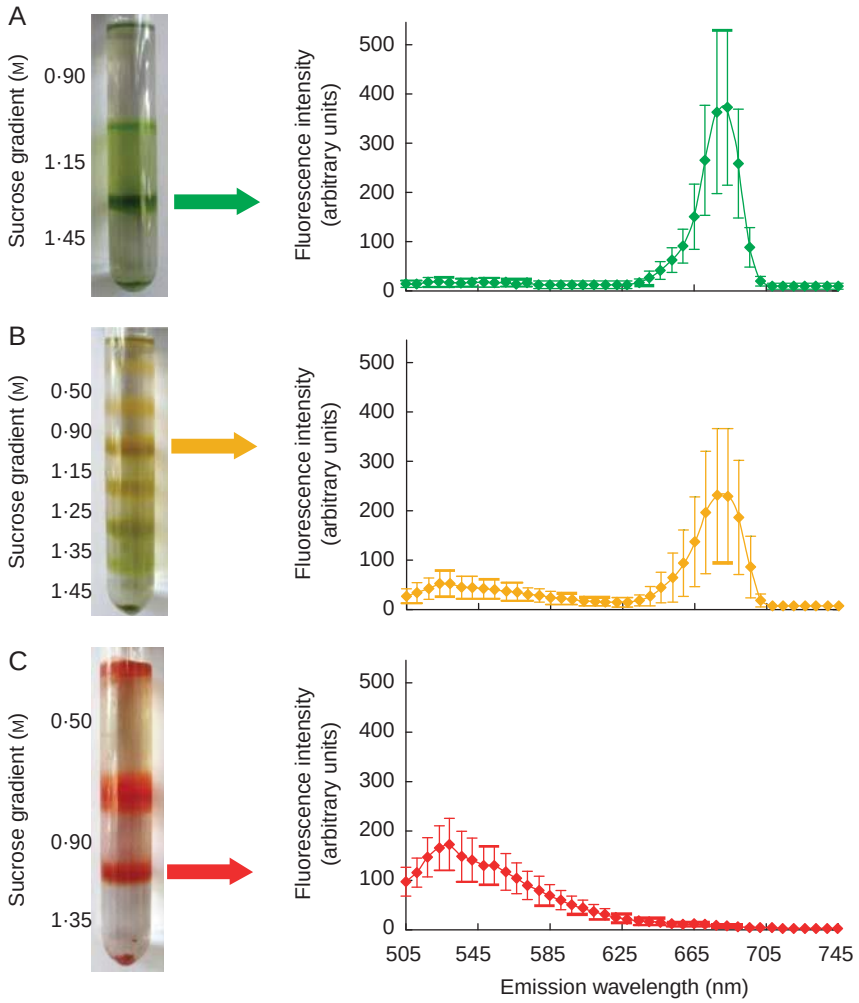

FIG. 1. Separation on a sucrose gradient of tomato plastids at three stages of fruit ripening and corresponding fluorescence emission spectra. Plastid fractions from tomatoes at the mature green (A), breaker + 2 d (B) and breaker + 10 d (C) stages have been analysed by a laser confocal scanner to generate fluorescence emission spectra of individual plastids. Fluorescence intensity of the fractions indicated by an arrow is given as arbitrary units ± s.d. (n = 50). The excitation wavelength was 488 nm. The peaks of fluorescence emission around 520 and 680 nm correspond to carotenoids and chlorophylls, respectively.

saturation at the end of the time-lapse recording, the photomultiplier was adjusted to a low level of amplification, corresponding to a low signal/noise ratio. This was not required for the chlorophyll channel, where a decrease was expected, and for which the signal was already high at the beginning of the time-lapse recording.

RESULTS AND DISCUSSION

Characterization of chloroplast to chromoplast transition in isolated plastids with a focus on the intermediate stages

Plastids were isolated at different development stages and separated on sucrose gradients. The degree of integrity of plastids in the fractions was analysed with a fluorescent dye, CFDA (Schulz et al., 2004). The intactness of plastid fractions retained for further analysis (indicated by arrows in Fig. 1A–C), reached between 85 and 90 %, 80 and 85 %, and 65 and 70 %, respectively. The upper layers in the gradients contained broken plastids.

At the mature green stage, intact green plastids were localized in a single band at the 1·15 – 1·45 M sucrose interface (Fig. 1A). Fluorescence confocal analyses of the plastid pool in this band indicated the almost exclusive presence of chlorophylls, characteristic of chloroplasts (Fig. 1A). This was confirmed by a spectrophotometric analysis of pigment absorbances (Supplementary Data Fig. S2).

Plastids isolated from fruit at the breaker stage were separated into four layers in a more fragmented sucrose gradient, excluding broken plastids in the upper part of the gradient above 0·9 M sucrose (Fig. 1B). The presence of different fractions of plastids with different colours from green to yellow clearly indicates that the differentiation process is not occurring synchronously. It is well known that chloroplasts have a greater density and a smaller size than chromoplasts (Rosso, 1968; Iwatzuki et al., 1984; Hadjeb et al., 1988). A good illustration of the decrease in density during the transition from

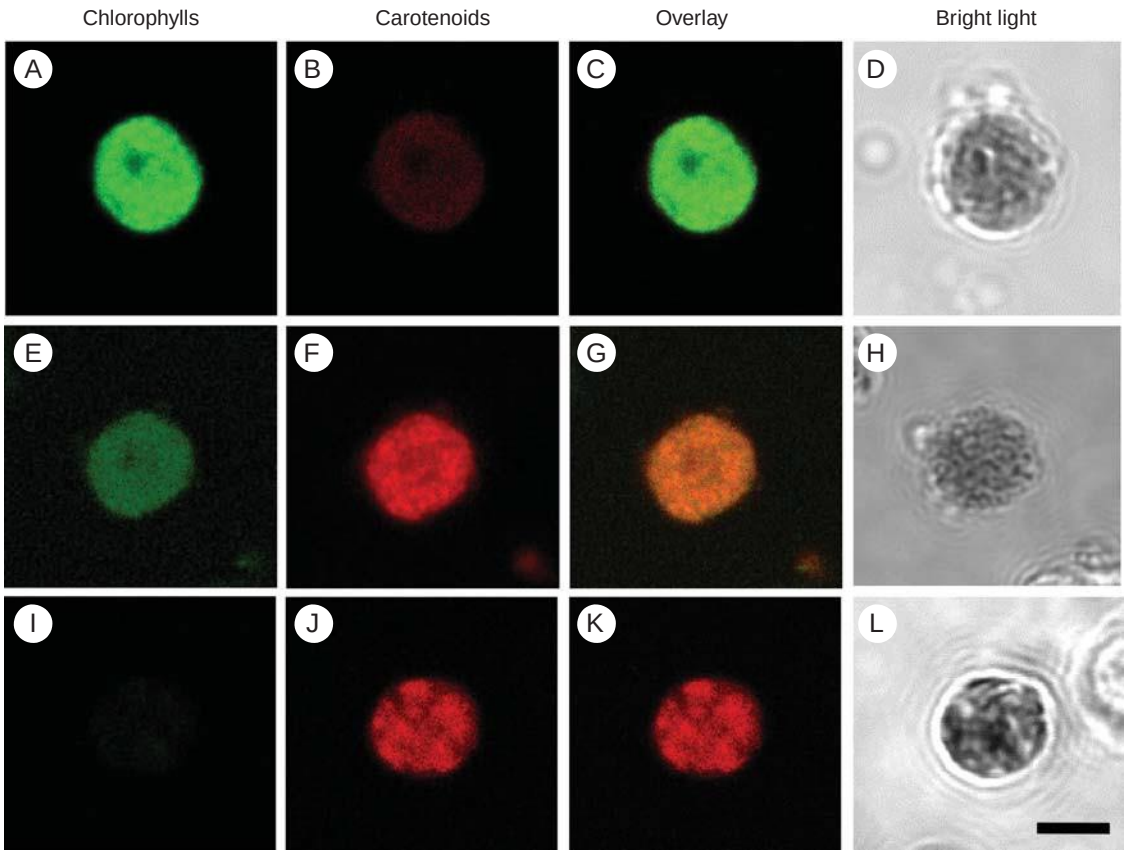

FIG. 2. Confocal microscopy of tomato plastids isolated at three stages of fruit ripening. (A–D) Chloroplasts isolated at the mature green stage. (E–H) Immature chromoplasts isolated at the turning stage (breaker + 2 d). (I–L) Chromoplasts isolated at the fully ripe stage (breaker + 10 d). (A), (E) and (I) correspond to chlorophyll fluorescence emitted from 650 to 750 nm; (B), (F) and (J) to carotenoid fluorescence emitted from 500 to 600 nm; and (C), (G) and (K) to the overlay of chlorophyll and carotenoid autofluorescence of the same plastid images. (D), (H) and (L) correspond to images of transmitted light. Chlorophyll fluorescence is shown in green and the carotenoid fluorescence is shown in red. Scale bar ¼ 4 mm.

chloroplasts to chromoplasts is shown in Fig. 1B, where a range of intermediate plastid forms are visible. After confocal microscopy analysis of the fractions, only the 0·9–1·15 M fraction contained a majority of plastids in a transient stage between chloroplast and chromoplast. Confocal analyses (Fig. 1B) showed that this fraction contained reduced levels of chlorophyll and significant amounts of carotenoids. This result was confirmed upon extraction of pigments and spectrophotometric analyses (Supplementary Data Fig. S2) and is typical for chromoplasts at the early stages of differentiation (also called immature chromoplasts). The other fractions below 1·15 M sucrose were more heterogeneous and comprised both early developing chromoplasts and chloroplasts (data not shown). Each band of the gradient was analysed for its chlorophyll and carotenoid content by solvent extraction (Supplementary Data Fig. S2) and the band at the interface of 0·9–1·15 M sucrose harboured a ratio close to 1:1 of chlorophylls:carotenoids. To our knowledge, intermediate plastids, in transition from chloroplast to the chromoplast stage, as shown in Fig. 2E – H, have never been characterized in previous works dealing with the isolation of chromoplasts from fruit pericarp (Siddique et al., 2006; Martı´ et al., 2009). Plastids from fully ripe fruit were separated in a single band at the 0·9– 1·35 M interface (Fig. 1C) and contained almost exclusively carotenoids, typical of chromoplasts.

Confocal observations of individual plastids provided more insight into the pigment transition at the plastid level (Fig. 2). The chloroplasts obtained from green tomatoes (Fig. 1A) were observed by confocal microscopy imaging, and a representative plastid is shown in Fig. 2A – D. It emitted fluorescence mostly in the chlorophyll emission range (Fig. 2A), and weakly in the carotenoid range (Fig. 2B), so that the carotenoid fluorescence emission is masked by chlorophyll fluorescence in the overlay picture (Fig. 2C). A typical plastid of the breaker stage (Fig. 2E – H), taken from the orange band of Fig. 1B, exhibited significant fluorescence of both chlorophylls (Fig. 2E) and carotenoids (Fig. 2F), giving an orange colour in the overlay picture (Fig. 2G). This plastid represented an intermediate differentiation stage between chloroplast and chromoplast. The suspension of chromoplasts isolated from red fruit contained only fully differentiated chromoplasts (Fig. 2I – L), that emitted fluorescence only in the carotenoid emission range (Fig. 2J), and not at the specific wavelength of chlorophylls (Fig. 2I). All isolated plastids were spherical; this may be due to the fact they were removed from the cell where it has been observed that stromules (Waters et al., 2004) and

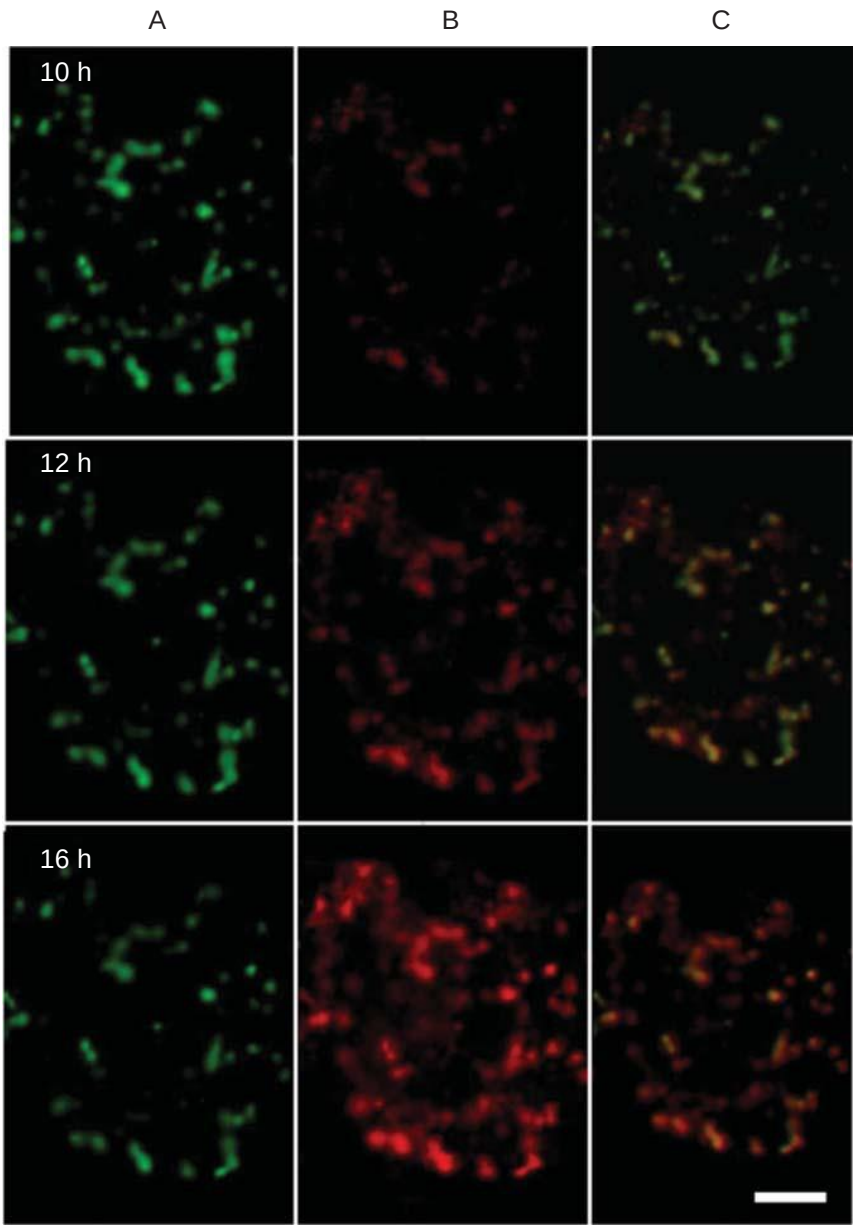

FIG. 3. Time-lapse evolution of chlorophyll and carotenoid fluorescence by confocal microscopy of plastids from tomato fruit mesocarp cells at the early stages of the transition from chloroplast to chromoplast. (A) Chlorophyll fluorescence, (B) carotenoid fluorescence and (C) overlay of both images at 10, 12 and 16 h. The emitted fluorescence was collected between 650 and 750 nm for chlorophyll and between 500 and 600 nm for carotenoid. The timing corresponds to the arrow shown in Fig. 4. A video showing the kinetics of these variations is available in supporting information (Supplementary Data, Video S1). Chlorophyll fluorescence is shown in green, and carotenoid fluorescence is shown in red. Scale bar ¼ 50 mm.

microfilaments and microtubules (Kwok and Hanson, 2003) control plastid morphology.

In situ real-time recording of the chloroplast to chromoplast transition

To get a complete sequence of the changes occurring at the beginning of the pigment switch, a real-time recording of mesocarp tissues was performed (Supplementary Data Video S1). Pictures extracted from the video (Fig. 3) show that the number of plastids per cell of the 'MicroTom' cultivar is around 70, which is about the number of plastids per cell observed in the inner mesocarp of 'Ailsa Craig' (Waters et al., 2004). However, there also appeared to be differences between the two cultivars. During the transition, the fluorescence of carotenoids increased steadily within the plastids within 6 h (Fig. 3B). Interestingly, in these conditions, where observations could be made at the cell level, it appeared that the transition was rather synchronous, as shown by the presence of a limited number of plastids having different colours upon merging of the spectral views of chlorophyll and carotenoids (Fig. 3C). This is in contrast to the large number of intermediate plastid forms observed at the breaker stage in the isolated plastid population arising from whole tissues

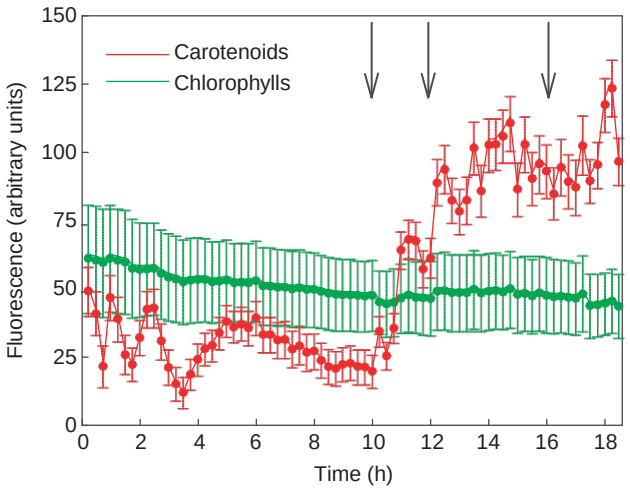

FIG. 4. In situ kinetics of chlorophyll and carotenoid fluorescence emission by confocal microscopy of plastids of tomato fruit mesocarp cells at the early stages of the transition from chloroplast to chromoplast. Intensity measurements were made on a selection of 150 individual plastids from four different cells. Error bars represent the s.d. The arrows show the timing of the pictures in Fig. 3.

(Fig. 1B). Although in the present experiment the ripening process was accelerated by the addition of ACC, it can be assumed that the synchrony of transition from chloroplasts to chromoplasts was higher within a cell than between cells of the fruit tissue.

The ratio of chlorophyll to carotenoid fluorescence was calculated for a total of 70 plastids present in a single cell at 10 and 16 h, by considering that a ratio < 1 corresponded to chloroplasts, and a ratio > 1 to chromoplasts. Over the 6 h of image recording, 84·3 % of the fluorescing plastids turned from chloroplasts to chromoplasts, 8·6 % stayed as chloroplasts and 7·1 % were already chromoplasts at the beginning of the observation period. Therefore, > 80 % of the plastids have undergone a transition within 6 h, which is indicative of strong intracellular synchrony. Interestingly, there was no appearance of new plastids, thus confirming that all chromoplasts derived from pre-existing chloroplasts, as suggested by Pyke and Howells (2002) and Waters et al. (2004).

The recording of fluorescence emission of chlorophyll and carotenoids showed that very little change occurred during the first 10 h (Fig. 4). At 10 h, the carotenoid fluorescence suddenly increased, while chlorophyll fluorescence decreased more slowly. The increase in carotenoid fluorescence was 2·5-fold in the first 20 h interval, while the decrease in chlorophyll fluorescence was at most 0·25-fold during the same period. After 14 h, the fluorescence of carotenoids reached a maximum and stayed constant until the end of the observation period. This is in contrast to whole fruit that continue to accumulate carotenoids over a week after the breaker stage (Fraser et al., 1994). The excision and survival of fruit tissues may be responsible for the early cessation of carotenoid accumulation beyond 14 h (Fig. 4); however, it is also possible that the cell has reached its maximum capacity of carotenoid accumulation. Indeed, it seems that the continuous accumulation of carotenoids in fruit tissues is the sum of carotenoid content of thousands of cells, which gradually turn red. The possibility that chlorophyll degradation products could exhibit fluorescence and therefore unmask chlorophyll breakdown should be excluded. It has been demonstrated that chlorophyll breakdown products do not accumulate in higher plants (Matile et al., 1999) and they are rapidly metabolized into non-fluorescent compounds (Oberhuber et al., 2003). Our data on pigment changes are in agreement with previous observations showing that lycopene accumulated at a rate that was between three and four times higher than the corresponding chlorophyll decline (Trudel and Ozbun, 1970; Wu and Kubota, 2008). The very fast increase in carotenoid accumulation can be related to a very strong increase in the expression of phytoene synthase and phytoene desaturase genes between the mature green and breaker stages (Giuliano et al., 1993; Ronen et al., 1999).

The biogenesis of plastids, more particularly the conversion of chloroplasts into chromoplasts, has become a major field of interest among plastid physiologists (Pyke, 2007; Kahlau and Bock, 2008; Egea et al., 2010). In the present work, laser scanning confocal microscopy has been used to study, at a subcellular resolution, the biogenesis of chromoplasts resulting from the conversion of chloroplasts in tomato fruit. The changes in carotenoids and chlorophylls have been monitored simultaneously on both isolated plastids and intact live mesocarp tissues. By recording the kinetics of short time changes, this method has allowed a fine description of the early steps of the transition to be described. The transition started in the nascent chromoplast by a sharp accumulation of carotenoids while the chlorophyll level was still high. The transition was more synchronous within plastids of a single cell than between cells of the fruit tissue. The real-time monitoring of the transition presented in a video revealed that all the chromoplasts derived from pre-existing chloroplasts in mesocarp tissues.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Video S1: pigment changes that occur during the early stages of the transition from chloroplast to chromoplast in mesocarp tomato cells, as viewed under a laser scanning confocal system (Leica LSCM-SP2) coupled with an upright microscope (Leica DM6000); the video is available in both .wmv and .avi formats. Figure S1: experimental device used to make the video. Figure S2: ratio of chlorophylls to carotenoids in isolated plastids.

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Chapter II-Proteomic analysis of chloroplast-to-chromoplast transition in tomato reveals metabolic shifts coupled with disrupted thylakoid biogenesis machinery and elevated energy-production components

Introduction

Fruit ripening involves a series of biochemical and physiological events resulting in organoleptic changes in texture, aroma and colour that make fruit attractive to the consumer. In many fruit, one of the most important and more visible changes corresponds to the loss of chlorophyll and the synthesis of colored compounds such as carotenoids. This happens through the transformation of chloroplasts into chromoplasts (Camara et al., 1995). In tomato which is widely used as a model fruit, the ripening process is triggered by the plant hormone ethylene (Lelièvre et al., 1997; Giovanonni, 2001). Fruit physiologists have contributed to the elucidation of some of the mechanisms governing the mode of action of ethylene and the accumulation of metabolites responsible for quality attributes (e.g. aromas, vitamins and antioxidants). A number of genes involved in the fruit ripening process have been isolated especially in the recent years with the use of high-throughput methods of genomics (Moore et al., 2002). However, little attention has been paid to the understanding of the mechanisms of fruit ripening at the sub-cellular level. For instance, the intimate mechanisms occurring in the chromoplasts are not well understood despite their crucial role in the generation of major metabolites that are essential for the sensory and nutritional quality of fruit.

A large majority of the proteins present in the chromoplast are encoded by the nucleus and therefore imported into the organelle. The chromoplast genome encodes for only few proteins participating in the build-up of the chromoplast structure and in house-keeping activities (Soll J., 2002). Important programmes devoted to the generation of

ESTs and the sequence of the tomato genome has been published recently (Reference). However for the reasons mentioned above, knowledge on gene expression and on genome sequences are of limited value for understanding the function of chromoplast in the synthesis of metabolites of interest. In addition, these programmes can take into account neither post-translational protein modifications nor subcellular localisation of the biosynthetic pathways. For these reasons, high-throughput proteomics associated with bio-informatics represents the most attractive and most suitable methodology for the understanding of the functions of the chromoplast. In turn, the knowledge of the tomato chloroplast/chromoplast proteome is expected to bring complementary information for the annotation of the tomato genome sequence.

In the present chapter we present our contribution to the quantitative proteomic analysis of the chloroplast-to-chromoplast transition in tomato fruit corresponding to a paper recently accepted in Plant Physiology for which I am a first co-author with equal participation to the work.

Foreword:

The undertaking of a proteomic program requires a wide range of expertise. In our consortium the proteomic analysis has been carried out by mass spectrometry using a LTQ-Orbitrap mass spectrometer at the Proteomic platform of the Toulouse Midi-Pyrénées Génopole (Michel Rossignol, Carole Pichereaux and David Bouyssié). Bio-informatics and statistical analysis of the data has been carried out in the host laboratory under the supervision of Mohamed Zouine and Elie Maza. I have personally participated in all biochemical and metabolic aspects of the work with special investment in: (1) the isolation of plastids in collaboration with Cristina Barsan and Isabel Egea; (2) the in-depth analysis of proteins involved in the structural modifications of plastids, provision of energy and translocation of precursors; (3) the immunoblot analysis of several proteins in order to validate the mass spectrometry quantifications.

Proteomic Analysis of Chloroplast-to-Chromoplast Transition in Tomato Reveals Metabolic Shifts Coupled with Disrupted Thylakoid Biogenesis Machinery and Elevated Energy-Production Components[1][W]

Cristina Barsan [2], Mohamed Zouine[2], Elie Maza[2], Wanping Bian [2], Isabel Egea, Michel Rossignol, David Bouyssie, Carole Pichereaux, Eduardo Purgatto, Mondher Bouzayen, Alain Latché, and Jean-Claude Pech *

Université de Toulouse, Institut National Polytechnique-Ecole Nationale Supérieure Agronomique de Toulouse, Génomique et Biotechnologie des Fruits, Castanet-Tolosan F–31326, France (C.B., M.Z., E.M., W.B., I.E., M.B., A.L., J.-C.P.); Institut National de la Recherche Agronomique, Génomique et Biotechnologie des Fruits, Chemin de Borde Rouge, Castanet-Tolosan F–31326, France (C.B., M.Z., E.M., W.B., I.E., M.B., A.L., J.-C.P.); Fédération de Recherche 3450, Agrobiosciences, Interactions et Biodiversités, Plateforme Protéomique Génopole Toulouse Midi-Pyrénées, Institut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique, F–31077 Toulouse, France (M.R., C.P.); Université de Toulouse, Université Paul Sabatier, Institut de Pharmacologie et de Biologie Structurale, Toulouse F–31077, France (M.R., D.B., C.P.); and Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Depto. de Alimentos e Nutrição Experimental, 05508–000 São Paulo, Brazil (E.P.)

A comparative proteomic approach was performed to identify differentially expressed proteins in plastids at three stages of tomato (Solanum lycopersicum) fruit ripening (mature-green, breaker, red). Stringent curation and processing of the data from three independent replicates identified 1,932 proteins among which 1,529 were quantified by spectral counting. The quantification procedures have been subsequently validated by immunoblot analysis of six proteins representative of distinct metabolic or regulatory pathways. Among the main features of the chloroplast-to-chromoplast transition revealed by the study, chromoplastogenesis appears to be associated with major metabolic shifts: (1) strong decrease in abundance of proteins of light reactions (photosynthesis, Calvin cycle, photorespiration) and carbohydrate metabolism (starch synthesis/degradation), mostly between breaker and red stages and (2) increase in terpenoid biosynthesis (including carotenoids) and stress-response proteins (ascorbate-glutathione cycle, abiotic stress, redox, heat shock). These metabolic shifts are preceded by the accumulation of plastid-encoded acetyl Coenzyme A carboxylase D proteins accounting for the generation of a storage matrix that will accumulate carotenoids. Of particular note is the high abundance of proteins involved in providing energy and in metabolites import. Structural differentiation of the chromoplast is characterized by a sharp and continuous decrease of thylakoid proteins whereas envelope and stroma proteins remain remarkably stable. This is coincident with the disruption of the machinery for thylakoids and photosystem biogenesis (vesicular trafficking, provision of material for thylakoid biosynthesis, photosystems assembly) and the loss of the plastid division machinery. Altogether, the data provide new insights on the chromoplast differentiation process while enriching our knowledge of the plant plastid proteome.

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[2] These authors contributed equally to the article.

* Corresponding author; e-mail pech@ensat.fr.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jean-Claude Pech (pech@ensat.fr).

[W] The online version of this article contains Web-only data.
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One of the most visible events occurring during fruit ripening is the loss of chlorophyll and the synthesis of colored compounds. In many fruit, such as the tomato (Solanum lycopersicum), the change in color from green to red is due to the differentiation of chloroplasts into chromoplasts and is accompanied by the accumulation of carotenoids.

Numerous studies have been devoted to ultrastructural events underlying the conversion of chloroplast to chromoplast. The events investigated include, in particular, the formation of protein-accumulating bodies and the remodeling of the internal membrane system. At the structural level, the differentiation of chromoplasts consists mainly of the lysis of the grana and thylakoids (Spurr and Harris, 1968) but also includes new synthesis of membranes derived from the inner plastid membrane and that become the site for the formation of carotenoids (Simkin et al., 2007).

At the biochemical and molecular level chromoplast differentiation studies have been largely dedicated to the synthesis of carotenoids (Camara et al., 1995; Bramley, 2002). However, although highly specialized, chromoplasts carry out a variety of functions, many of them persisting from the chloroplast (Bouvier and Camara, 2007; Egea et al., 2010). The biochemical and structural events during chromoplast differentiation have been reviewed in a number of articles over the last decades (Thomson and Whatley, 1980; Ljubesić et al., 1991; Marano et al., 1993; Camara et al., 1995; Waters and Pyke, 2004; Lopez-Juez, 2007; Egea et al., 2010; Bian et al., 2011).

In the recent years high-throughput technologies have provided novel and extensive information on the plastid proteome of higher plants with strong emphasis on the chloroplast (for review, see van Wijk and Baginsky, 2011). Less information is available on the chromoplast proteome to the studies focusing on pepper (Capsicum annuum; Siddique et al., 2006), tomato (Barsan et al., 2010), and sweet orange (Citrus sinensis; Zeng et al., 2011). A comparison of the chromoplast proteome of sweet orange and tomato shows a high level of conservation although some specificities have been encountered (Zeng et al., 2011). Nevertheless, the global changes occurring during the differentiation of chromoplasts from chloroplasts have not been evaluated so far. In this work, a quantitative proteomic analysis was carried out to understand the regulation of the metabolic and structural changes occurring in tomato fruit plastids during the transformation of chloroplasts into chromoplasts.

RESULTS AND DISCUSSION

Inventory of Proteins Present in Tomato Fruit Plastids during the Chloroplast-to-Chromoplast Transition

The experimental design used for fruit sampling and biological replications is presented in Figure 1. Plastids from four sets of about 100 g of pericarp (corresponding to 25–30 fruits) were isolated separately in three independent replicates (Rep 1–3) for each developmental stage, mature green (MG; 1–3), breaker (B; 1–3), and red (R; 1–3). This procedure was repeated twice. Two protein extracts from four individual plastid fractions were pooled for each replicate. The raw data coming from the analysis of the replicates of the tomato plastid proteins were curated by comparing the set of proteins with five databases (AT-CHLORO, Plprot, PPDB, SUBA, and Uniprot) and three predictors for subcellular localization (TargetP, Predotar, iPSORT). Only proteins present in at least two databases or predicted to be plastid localized by one of the predictors were retained for analysis. In addition manual curation was performed on the basis of the information available in the literature. By combining the curated list of proteins encountered in the three replicates of the tomato plastids

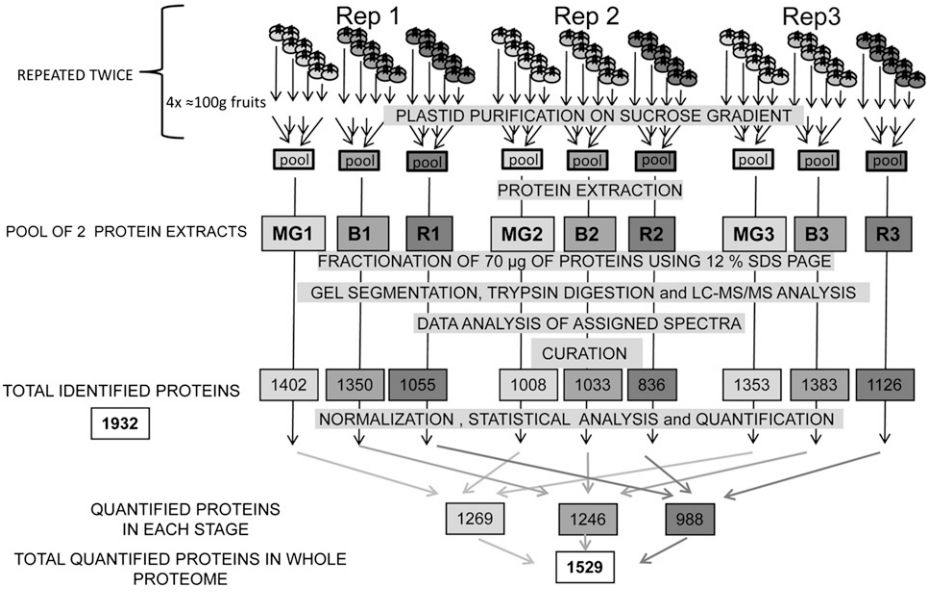


Figure 1. Summarized experimental design used in this proteomic study. Plastids from four sets of four fruits were isolated separately in three independent replicates (Rep1, Rep2, and Rep3) for each developmental stage (MG 1–3; B 1–3; and R 1–3). This procedure was repeated twice and plastids corresponding to each stage were pooled for each experiment before protein extraction. The different steps from purification of plastids to curation of data are indicated and described in detail in "Material and Methods." The number of proteins encountered in each individual analysis is given under the denomination of total resources, resulting in a nonredundant total list of 1,932 proteins that are reported in Supplemental Table S1. After normalization and statistical analysis, the number of proteins that could be quantified is indicated under the denomination of total quantified proteins. The list and quantitative information of the 1,529 quantified proteins is given in Supplemental Table S2.

at three stages of development, an inventory of 1,932 proteins (including 47 proteins encoded by the plastid genome) was drawn up and cataloged in Supplemental Table S1. Previous work with fruit chromoplasts identified 988 proteins in tomato (Barsan et al., 2010), 151 in pepper (Siddique et al., 2006), and 493 in sweet orange fruit (Zeng et al., 2011). The inventory reported here is in the same range of magnitude as other plastids database: 1,345 in AT-CHLORO, 2,043 in plprot, 1,367 in PPDB, and 2,112 in SUBA. Our work brings 362 proteins that had not yet been referenced in plastid databases and that are predicted to be plastid localized by at least one predictor. Among these, 38 had already been cataloged in the tomato chromoplast proteome (Barsan et al., 2010). The size of the plant plastid proteome has been evaluated by applying a combination of chloroplast transit peptides predictors programs to the screening of the nucleus-encoded amino acid sequences available in databases. In Arabidopsis (Arabidopsis thaliana) predictions vary between 2,100 (Richly and Leister, 2004) and 2,700 (Millar et al., 2006). Armbruster et al. (2011) come to similar predictions of between 2,000 and 3,000 proteins. In rice (Oryza sativa), predictions estimate at 4,800 the number of chloroplast proteins (Richly and Leister, 2004). Therefore it seems that marked differences exist between plant species. However discrepancies are observed in the estimation of the size of the plastid proteome probably due to the use of different evaluation methods and to the fact that many plastid proteins are not predicted by prediction tools (Kleffmann et al., 2006). By combining three different approaches for protein localization (experimental determination by cell biology methods, homology-based identification, and predictions by targeting programs), Pierleoni et al. (2007) estimated the Arabidopsis plastid proteome size at 4,875 proteins. With the availability of the tomato sequence genome, we have been able to calculate that the number of proteins predicted to be plastidial in the tomato genome is of 2,696, 4,651, and 4,142 using Predotar, iPSORT, and TargetP, respectively. Therefore it can be concluded that the present inventory of plastid proteins in databases is probably not exhaustive.

The number of proteins of the tomato plastid proteome referenced in each of the five databases is presented in Figure 2A. It indicates that the overlap is higher than 50% for SUBA (55.7%) and Uniprot (63.3%), more than 35% for Plprot (35.3%) and AT-CHLORO (44.2%). PPDB has the lowest overlap with 23.9%. The percentage of tomato proteins that are not referenced in any of the five databases is 22.5% only while the percentage referenced at least one, two, three, four, and five times is 77.5%, 56.9%, 44.9%, 28.3%, and 13.9%, respectively (Fig. 2B). A large majority of proteins (1,492 = 77.2%) were predicted to be plastid localized by at least one prediction software. The overlap of tomato plastid proteins predicted by the three softwares is presented in Figure 3. TargetP, iPSORT, and Predotar forecast a plastid localization of 45.4% (876 proteins), 37.9% (733), and 40% (734) proteins, respectively. The percentage of proteins predicted by all three programs is only 7.3% (142). Previous

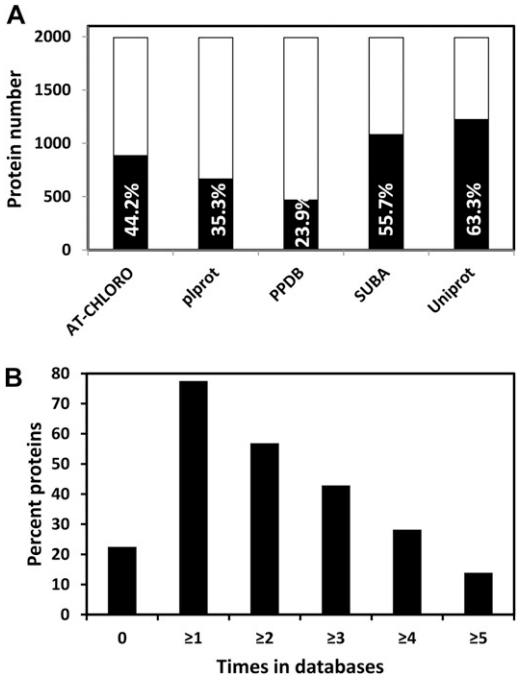


Figure 2. Proteins of the tomato plastid proteome referenced in five plastid databases. A, Number and percentage of proteins (in black bars) referenced in each of the five databases, calculated on the basis of the 1,932 proteins listed in Supplemental Table S1. B, Percentage of the tomato plastid proteins not referenced (0) or referenced at least one, two, three, four, and five times in the different databases. The five databases are the following: AT-CHLORO, Plprot, PPDB, Uniprot, and SUBA.

reports have indicated similar proportions of mitochondrial proteins predicted in common by four prediction programs (Heazlewood et al., 2004). Predotar and TargetP were the closest in terms of predictions with 485 proteins in common. It is noticeable that predictions specific to iPSORT were the highest with 509 proteins (Fig. 3). Predictions made by the three programs within the existing chloroplast databases are extremely variable from 83% with TargetP in PPDB to 43% with iPSORT in SUBA (Table I).

The inventory of tomato fruit plastid proteins present at different stages of plastid differentiation enriches the knowledge of the plant plastid proteome. Most of the data available were related to chloroplasts (Armbruster et al., 2011; van Wijk and Baginsky, 2011), although some information was also available for nonphotosynthetic plastid structures such as amyloplasts (Andon et al., 2002; Balmer et al., 2006), etioplasts (von Zychlinski et al., 2005), proplastids (Baginsky et al., 2004), and embryoplasts (Demartini et al., 2011). The present inventory also provides information that complements previous articles published on the chromoplast proteome (bell pepper: Siddique et al., 2006; tomato: Barsan et al., 2010; and sweet orange: Zeng et al., 2011).

The plastid proteome of tomato fruit at the MG stage was compared with the proteome of Arabidopsis

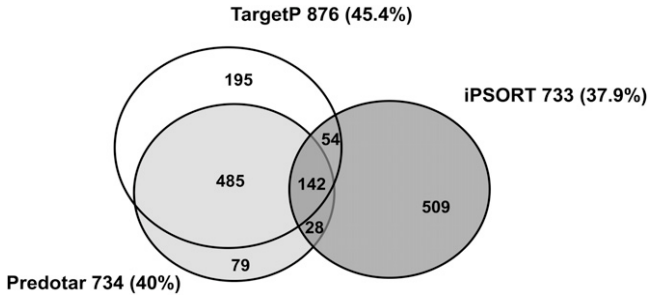


Figure 3. Venn diagram of the number of proteins of the tomato plastid proteome predicted to be plastid localized by three predictors. A total of 1,492 proteins are predicted by at least one predictor, TargetP, Predotar, or iPSORT. The 47 plastid-encoded proteins are not concerned.

chloroplasts reported in the AT-CHLORO database (Ferro et al., 2010) and in Zybailov et al. (2008). It appears that the percentage of proteins classified according to the MapMan functional classes is very similar in the two proteomes, particularly for the class corresponding to photosynthesis (Supplemental Fig. S1). This indicates that MG plastids have the general characteristics of chloroplasts although fruit photosynthesis is very low compared with leaf photosynthesis (Blanke and Lenz, 1989; Hetherington et al., 1998) and plays an unimportant role in fruit metabolism (Lytovchenko et al., 2011).

Proteomic Specificities of the Three Stages of Plastid Differentiation in Terms of Protein Abundance

After normalization and statistical analysis, 1,529 proteins out of the inventory of 1,932 were quantified and their pattern of abundance determined (Fig. 1; Supplemental Table S2). A comparison of the protein pattern at different stages two by two is presented as scatter plots in Figure 4. Between the MG and B stages (Fig. 4A) a large number of proteins underwent no significant change in abundance (975 = 63.7%). Only 17 proteins were significantly more abundant at the green stage and 28 at the B stage, while 75 proteins are specific to the MG stage and 89 to the B stage. Between the B and R stages 797 (52.1%) proteins underwent no quantitative changes. Among proteins showing differences in abundance, 43 are more abundant at the B stage and 15 at the R stage while 182 and 43 are specific to the B and R stages, respectively (Fig. 4B). Comparing the two most distant stages (MG and R), the number of proteins undergoing no changes between the two stages was found to be only 578, representing 37.8% of the proteome. Therefore around 60% of the proteins change in abundance during the whole differentiation process (Fig. 4C) with 134 proteins overexpressed in the MG and 92 in the R plastids. The number of proteins encountered at one stage only was 286 in the MG and 86 in the R plastids (Fig. 4C).

Proteomic data on the whole tomato fruit are available in which quantitative data on plastidial proteins can be encountered (Rocco et al., 2006; Faurobert et al., 2007). Forty-seven proteins have been identified both in the plastid proteome described in this work and in the whole fruit proteome of Rocco et al. (2006) and Faurobert et al. (2007). Although the tomato varieties and the quantification methods were very different, the ratios of abundance between the three stages of plastid development (MG, B, and R) were identical or similar, thus confirming the accuracy of the quantification methods.

Changes in Abundance of Proteins Encoded by the Plastid Genome

Among the 87 proteins predicted to be encoded by the plastid genome, 47 were encountered in our study (Supplemental Table S1) and the abundance of 32 of them is presented in Figure 5 for all three stages of plastid development (Supplemental Table S). These comprise several proteins participating in photosynthesis whose abundance remains essentially stable between the MG and B stages but disappear or strongly decrease at the R stage when the photosynthetic system is dismantled: two proteins of PSI, five of PSII, three of PS cytochrome b6, and five of PS ATP synthase. Only one protein of PSII (PSBC), and two proteins of cytochrome b6 (PETA and PETB) are present at the R stage at substantial amounts. Immunoblots were performed for the PSAD protein of PSI and PSBA/D of PSII (Fig. 6). They are decreasing in abundance between the MG and B stages and are to- tally undetectable at the R stage, which is globally in agreement with the proteomic analysis. Surprisingly all the six ATP synthase subunits encoded by the plastid

Table I. Number of proteins predicted by three predictors in the tomato plastid proteome and in four plastid databases

Predictions are made only on nuclear-encoded proteins. For plprot and PPDB, only the subset of Arabidopsis plastidial proteins was considered (indicated by an asterisk).

Database	No. of Nuclear-Encoded Proteins	TargetP	iPSORT	Predotar
Tomato plastid	1,885	876 (46.5%)	733 (38.9%)	730 (38.7%)
AT-CHLORO	1,345	928 (68.9%)	660 (49%)	764 (56.8%)
plprot*	1,006	493 (49.0%)	489 (48.6%)	546 (54.3%)
PPDB*	1,178	980 (83.2%)	771 (65.4%)	908 (77.1%)
SUBA	2,112	1,208 (57.1%)	913 (43.2%)	1,039 (49.2%)

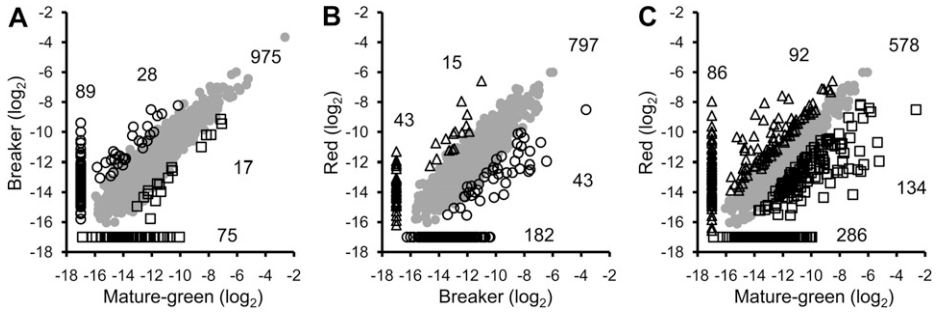


Figure 4. Scatter plots comparing $\log_2$ protein abundance between two differentiation stages of tomato fruit plastids. A, MG versus B. B, B versus R. C, MG versus R. Shades of gray represent proteins that are equally abundant in both stages; open squares correspond to proteins overexpressed at the MG stage; open circles are proteins overexpressed at the B stage, and open triangles represent proteins overabundant at the R stage. Data along the axis of the scatter plots indicate proteins that have been quantified at one of the two stages only and totally absent at the other stage. Numbers correspond to the number of proteins in each category. To draw the graphs, a $\log_2$ value of 217 was arbitrarily affected to proteins for which no abundance value was available in Supplemental Table S2.

genome were found. They all undergo a continuous decrease in abundance, but are still present at significant amount at the R stage, indicating that the ATP synthesis machinery is maintained at a good level throughout chromoplast development. The large subunit of Rubisco (RBCL) continuously decreases in abundance but is present at significant levels in red plastids (Fig. 5). The pattern of changes of RBCL is identical when evaluated by western blot (Fig. 6), thus providing another confirmation of the reliability of the quantification procedure. Another interesting information given in Figure 5 is that the sum of ribosomal proteins of the small 30S subunit (RPS) and of the large 50S subunit (RPL) strongly decline in abundance, with the RPL proteins absent at the R stage. This is in agreement with the gradual down-regulation of plastid translation observed by Kahlau and Bock (2008) during chromoplast differentiation in tomato. In contrast, the only caseinolytic protease encoded by the plastid genome, CaseinoLytic Plastidial Protease1 (CLPP1) is not changing in abundance, indicating a high level of protein processing throughout the differentiation process. This is in line with the sustained abundance of elements of the protein import machinery discussed below. In our study special attention has been given to the acetyl CoA carboxylase (ACCD) protein involved in fatty acid biosynthesis because previous work has shown that chromoplast gene expression largely serves the production of ACCD (Kahlau and Bock, 2008). In our proteomic analysis it is decreasing in abundance, although at a significance of P = 0.06 only (Supplemental Table S2). Western blot of ACCD also shows a decrease in abundance between the MG and R stages (Fig. 6). This is in apparent contrast with the western-blot data of Kahlau and Bock (2008) showing an increase of the ACCD protein between the green and the turning stages. To explain the discrepancy, we

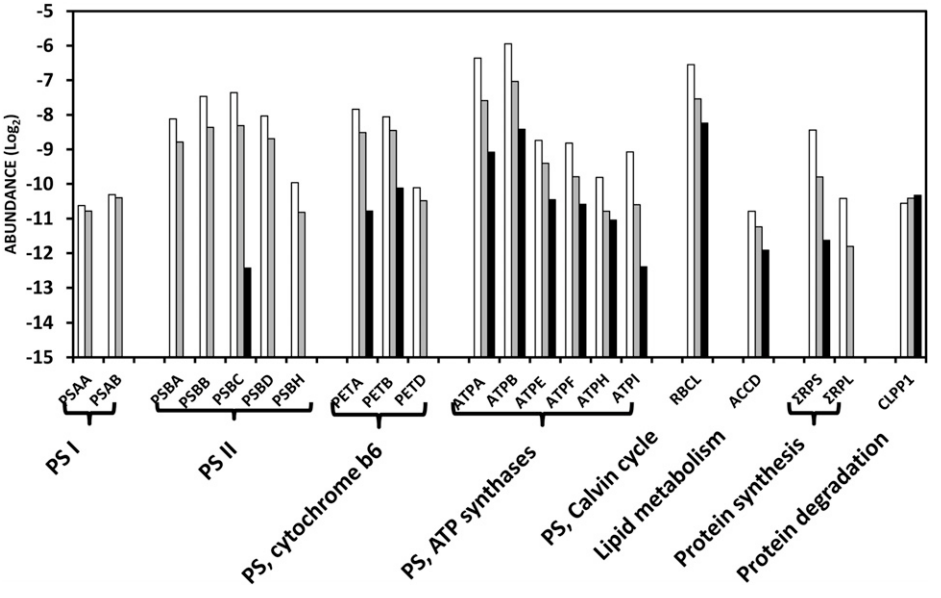


Figure 5. Abundance of proteins encoded by the plastid genome. Proteins present at all three stages of plastid development (MG: white bars; B: gray bars; and R: black bars) were classified according the MapMan functional classes. Protein abundance is expressed as a $\log_2$. The graphs were generated using the data and symbols of Supplemental Table S2. PSA, PSB, PET, and ATP correspond to proteins of the subunits of PSI, PSII, cytochrome b6, and ATP synthase complexes, respectively. RBCL corresponds to the large subunit of Rubisco, ACCD to the D subunit of acetyl CoA carboxylase, and CLPP1 to the caseinolytic protease P1. SRPS and SRPL represent the sum of proteins of the small 30S and the large 50S subunits, respectively.

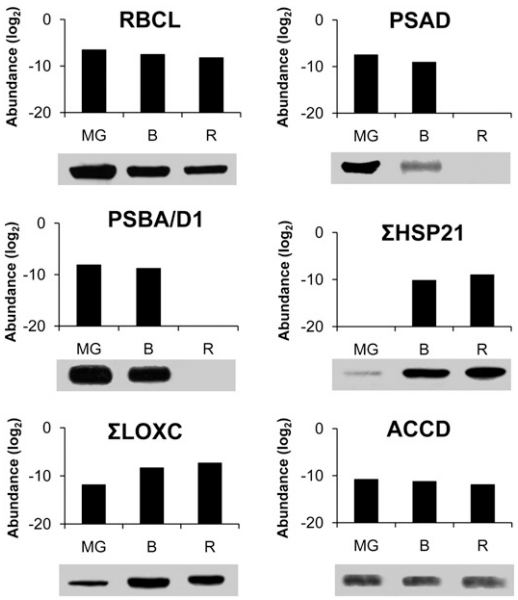


Figure 6. Comparison of protein abundance determined by proteomic analysis and immunoblotting. The abundance of proteins determined by proteomic analysis is expressed as $\log_2$. RBCL corresponds to the large subunit of Rubisco (GI89241679), PSAD to the D subunit of PSI (Solyc06g054260), PSBA/D1 to the A/D1 subunit of PSII (GI89241651), HSP21 to Heat Shock Proteins21 (Solyc03g082420 and Solyc05g014280), LOXC to lipoxygenase C (Solyc01g006540, Solyc01g006560, and Solyc12g011040), and ACCD to the D subunit of acetyl CoA carboxylase (GI89241680). For western blots, proteins were extracted from partially purified plastids as indicated in "Material and Methods."

performed another series of immunoblots blots by including an earlier stage of fruit development. The tendency observed in Figure 6 of a decline of ACCD protein between the MG and R stages was confirmed, but, most importantly the abundance of the protein was much lower in immature green fruit (Supplemental Fig. S2). These data indicate that the green fruit of Kahlau and Bock (2008) were probably sampled well before the MG stage. In our conditions, the MG stage was selected because the fruit has gained the capacity to ripen and to respond to the plant hormone ethylene (Pech et al., 2012) but plastids still have a chloroplastic structure with high chlorophyll, low carotenoid content, and absence of lycopene. Western blots of the ACCD protein indicate that important changes occur in plastids between the green and MG stages of development (Fig. 6). Whether these changes are part of the chromoplast differentiation process may be a matter of discussion. However, a unified view could be that plastid differentiation is a continuous process during fruit development in which the final steps of the differentiation corresponding to the chromatogenesis process per se are triggered by the plant hormone ethylene. In such a scheme, the early accumulation of the ACCD protein that is involved in fatty acid biosynthesis can be considered as a prerequisite for chromoplast differentiation by providing a storage matrix for the accumulation of carotenoids.

Changes in Subplastidial Compartmentation

The tomato plastid proteome referenced in Supplemental Table S2 has been screened with the AT_Chloro database (Ferro et al., 2010) to isolate proteins present in the stroma, thylakoids, and envelope membrane (Supplemental Table S3) and with the list of proteins of the plastoglobules established by Lundquist et al. (2012). In agreement with the structural remodeling of the internal membrane system (Spurr and Harris, 1968), this study clearly shows that the abundance of thylakoid proteins fell mostly during the transition from B to R stages while the abundance of proteins of the envelope and of the plastoglobules remained essentially unchanged and proteins of the stroma underwent a slight decrease in abundance (Fig. 7). The observation that the plastoglobule proteins underwent no changes during the transition from chloroplasts to chromoplasts is in line with the fact that the bulk of the carotenoids of tomato fruit are stored predominantly in the form of lycopene crystalloids in membrane-shaped structures (Harris and Spurr, 1969).

Kinetics of Changes in the Functional Classes during the Chloroplast-to-Chromoplast Transition

The kinetics of changes in protein abundance occurring during the three stages of chromoplast development have been classified into seven categories: stable, decreasing early, decreasing late, decreasing continuously, increasing early, increasing late, and increasing continuously (Table II). Among the seven categories, proteins whose abundance remained statistically constant slightly outweighed (569) proteins undergoing an increase in abundance (104 + 289 + 186 = 547). Those of the increasing category are the less abundant (72 + 82 + 100 = 254). Proteins showing a late decrease are the

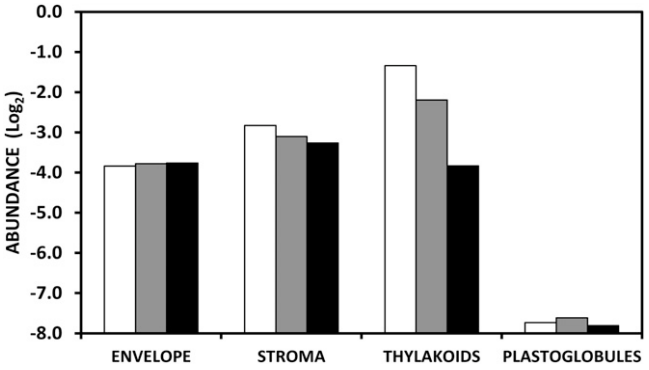
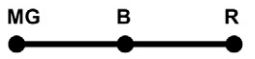
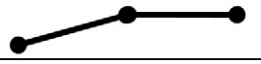


Figure 7. Abundance of proteins in the subplastidial compartments of tomato fruit plastids. Plastids were isolated from MG (white bars), B (gray bars), and R (black bars) fruit. Protein abundance is expressed as a $\log_2$. The present graph corresponds to Supplemental Table S3 generated by screening the tomato plastid proteome on the base of AT homologs with the AT-CHLORO subplastidial database (Ferro et al., 2010) for stroma, thylakoids, and envelope proteins and in Lundquist et al. (2012) for plastoglobule proteins.

Table II. Abundance pattern of the tomato plastid proteins classified into seven categories

Some proteins (127) for which we were unable to establish a consistent and logical pattern of abundance have been omitted. Data are from Supplemental Table S2.

Pattern	Denomination	No.
	Stables	569
	Decreasing continuously	104
	Decreasing late	289
	Decreasing early	186
	Increasing continuously	72
	Increasing late	82
	Increasing early	100

second-most numerous, indicating that important changes occur during the last step between B and R stages (Table II).

Among proteins showing the same abundance during the whole differentiation process, some functional classes were seen to be more stable than others (Fig. 8A). Sulfur assimilation, although represented by few proteins, is 100% stable, followed by tricarboxylic acid (TCA)/organic acid (85.3%), metal handling (66.7%), electron transport/ATP synthesis (62.5%), and glycolysis (56.5%). Representatives of classes comprising around one-third of stable proteins only are: amino acid metabolism, protein synthesis, lipid metabolism, secondary metabolism, Calvin cycle, and oxidative pentose phosphate pathway (OPP). Some classes have a very low percentage of stable proteins such as major carbohydrate (CHO; 10.7%), hormone metabolism (6.3%), and photosynthesis (2.4%). This picture allows the identification of a basal background of functions and structures that are roughly maintained during the differentiation of chromoplasts as well as profound changes in some functions that contribute to redirecting the plastid metabolism.

Photosynthesis is the most representative of the functional classes showing decreasing abundance during the chromoplast differentiation process with a much higher percentage of proteins (60.7%) decreasing late between the B and R stages (Fig. 8C) than decreasing early (17.9%; Fig. 8B) or continuously (15.5%; Fig. 8D). Tetrapyrrole biosynthesis (which comprises the biosynthesis of chlorophylls), OPP, and posttranslational event classes follows the same trend with a larger proportion of proteins undergoing a decline in the later stages (Fig. 8C). The situation where a high percentage of proteins decrease early is represented by the cofactors/vitamin metabolism, nitrogen metabolism, and biodegradation of xenobiotics classes with a decrease between the MG and B stages ranging from 25% to 36.4% of the proteins (Fig. 8B). The major CHO metabolism class comprises proteins decreasing in abundance in about the same proportions in the early (25.0%) and late (25.0%) stages of differentiation (Fig. 8, B and C).

The proportion of proteins showing increasing abundance (Fig. 8, E–G) in the different classes is generally low as compared with the decreasing categories (Fig. 8, B–D). One noticeable exception is proteins involved in fermentation of which 66.7% increase continuously (Fig. 8G). Interestingly stress-related proteins are those showing the highest percentage of increase with 15.7% (Fig. 8G), 15.7% (Fig. 8E), and 9.8% (Fig. 8F) in the increasing continuously, early or late categories, respectively. The significant proportion of cell division/organization proteins (15.1%) in the increasing continuously category (Fig. 8G) may account for the structural changes occurring in the formation of chromoplasts. Another remarkable observation is the high percentage (~30%) of hormone-related and DNA-related proteins showing an early (Fig. 8E) and a late (Fig. 8F) increase in abundance, respectively. The changes in hormone-related proteins are correlated to the increase in the synthesis of some hormones, mainly between B and R stages, such as abscisic acid (Zhang et al., 2009) and jasmonates (Fan et al., 1998). A small proportion of proteins of the major metabolisms, such as lipid, major and minor CHO, and OPP still underwent an increase

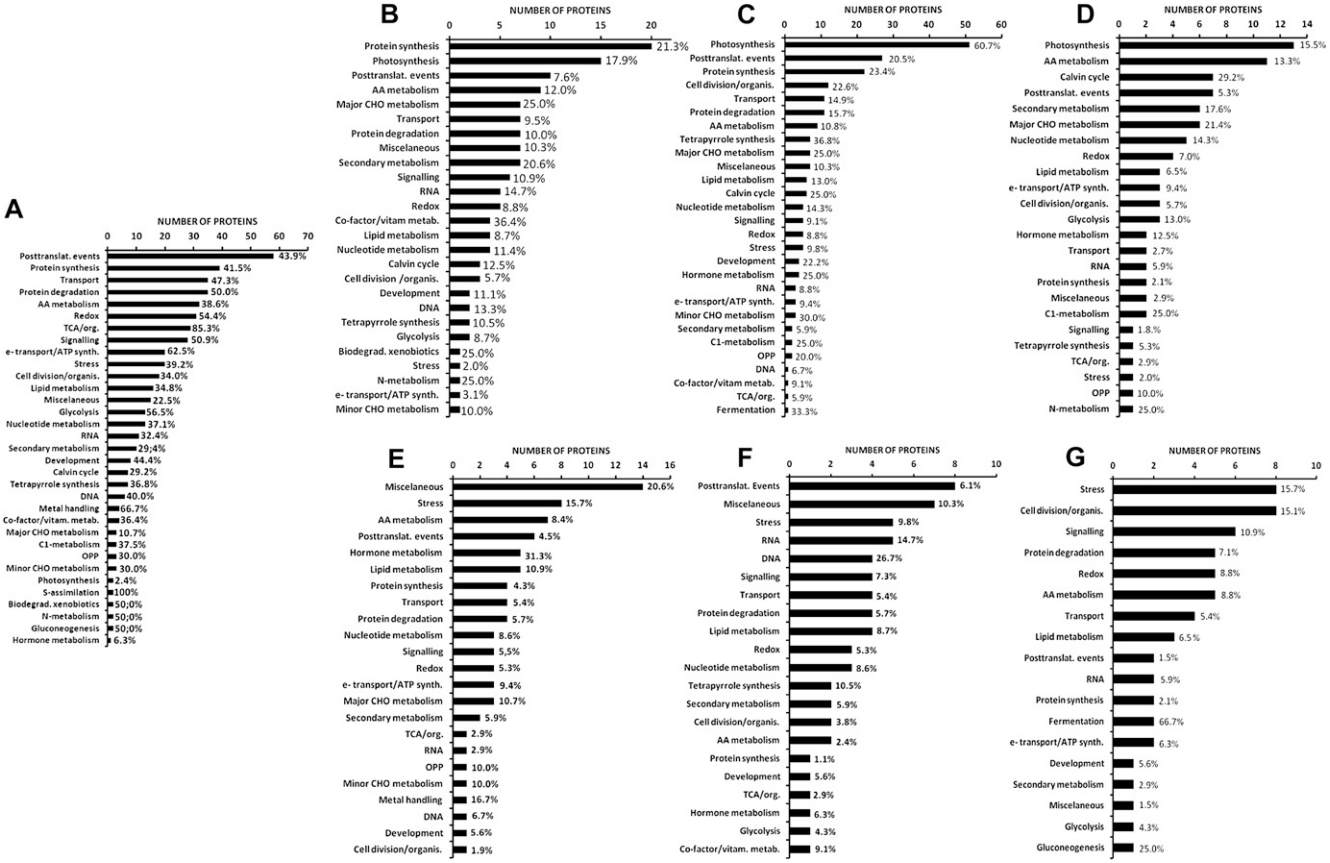


Figure 8. Number and percentage of proteins in the MapMan functional classes for seven patterns of abundance. A, Stable. B, Decreasing early. C, Decreasing late. D, Decreasing continuously. E, Increasing early. F, Increasing late. G, Increasing continuously. The abundance patterns are described in Table II. Numbers in % represent the percentage of proteins within each functional class.

in abundance. Noteworthy is the absence of proteins of the photosynthesis and Calvin cycle classes in the increasing categories (Fig. 8, E–G), a very low proportion of them remaining at constant amounts throughout chromoplast differentiation (Fig. 8A). Interestingly, two carbonic anhydrases of the TCA/organic acid class (Supplemental Table S2) were present at increasing levels (Solyc02g067750 and Solyc05g005490). Carbonic anhydrases have been found associated with the Rubisco complex (Jebanathirajah and Coleman, 1998). Rubisco being still present at late stages of chromoplast formation, carbonic anhydrases may contribute to the provision of CO_2 to Rubisco by catalyzing the dehydration of HCO_3^- in the alkaline stroma in close proximity to Rubisco. Some of the proteins increasing in abundance participate in the development of the sensory quality of the fruit, such as lipoxygenase C (LOXC; Solyc01g006540, Solyc01g006560, and Solyc12g011040), which is responsible for the biosynthesis of fatty-acid-derived aroma volatiles (Chen et al., 2004). The protein undergoes continuous increase from the MG to the R stages in proteomic analysis (Supplemental Table S2) as well as in western blots (Fig. 6). The increase in LOXC is coincident with the increase in the production of aroma volatiles (Birtić et al., 2009).

Overview of Metabolic and Regulatory Changes Occurring during Chromoplastogenesis

A heatmap showing the percentage of proteins in each functional class according to their abundance patterns allows various clusters to be distinguished (Fig. 9) Two clusters comprise a high percentage of stable proteins: III (sulfur assimilation and TCA/organic acid classes), IVd (redox, signaling, protein degradation, transport, electron transport/ATP synthesis, glycolysis, biodegradation/xenobiotics, metal handling, and gluconeogenesis). Two clusters include a majority of decreasing proteins corresponding to cluster II (Calvin cycle, major CHO metabolism and photosynthesis) and to cluster IVa (protein synthesis, cofactor/vitamin metabolism, tetrapyrrole synthesis, C1 and nitrogen metabolism). Cluster I is the only one comprising proteins with a strong increase in abundance, essentially fermentation. Clusters IVb and IVc have an almost equal percentage of proteins with stable, decreasing, and increasing patterns, indicating profound redirections in the corresponding functional classes, for instance OPP, metabolism of nucleotides, amino acids, and lipids as well as RNA synthesis.

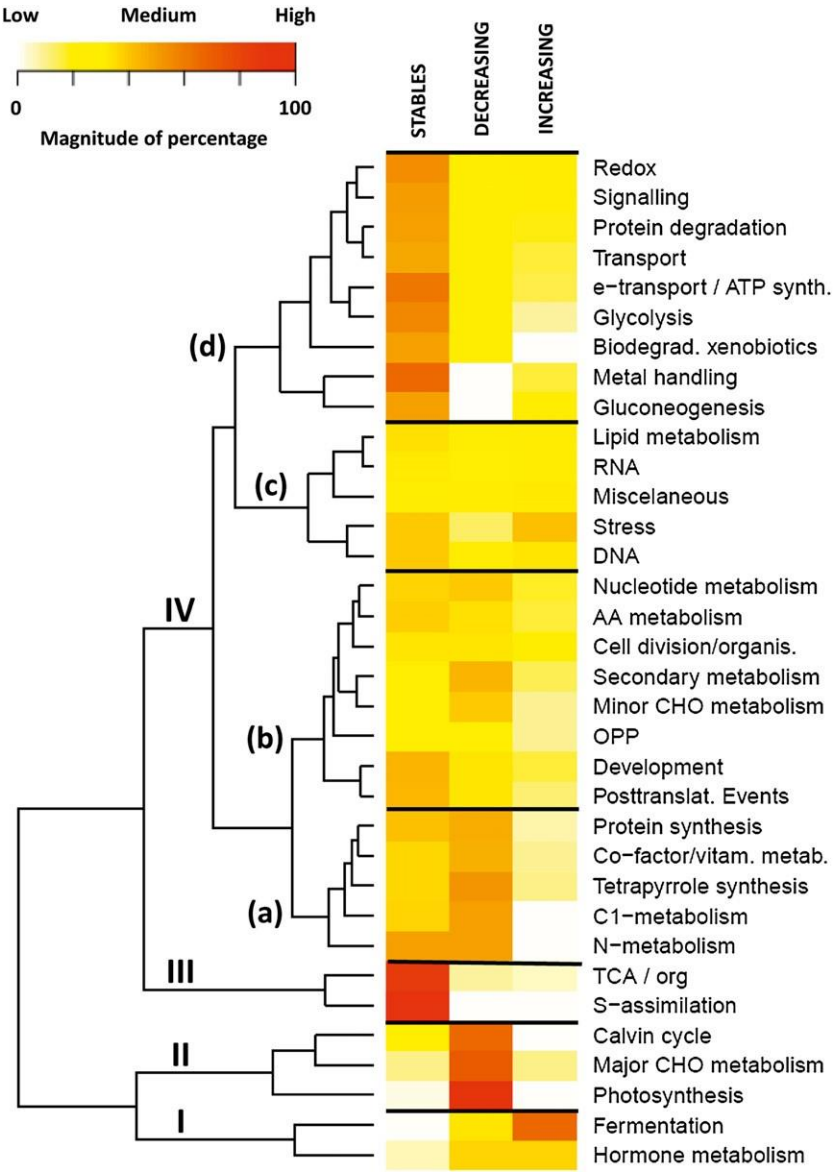


Figure 9. Heatmap showing the percentage of proteins of each functional class according to the abundance pattern. The three abundance patterns considered during the differentiation of chromoplasts are: stable, decreasing, and increasing. The magnitude of the percentage is represented by a color scale (top left) going from low (white), to medium (yellow), and high (red).

Changes in the abundance of individual proteins participating in the central metabolism are represented in a MapMan metabolic display (Fig. 10A). This illustrates the major shifts in metabolism occurring during the differentiation of chromoplasts. A large number of proteins involved in light reactions (including photosynthesis, Calvin cycle, and photorespiration) and major CHO metabolism (starch metabolism) are more abundant in the MG plastid and therefore decrease in abundance during chromoplastogenesis. Many of the proteins involved in the provision of energy to the plastid remain unchanged (TCA cycle, glycolysis, and electron transport/ATP synthesis). Some proteins increase in abundance such as carbonic anhydrases and some proteins involved in the metabolism of terpenoids (including carotenoids), lipids, amino acids, and ascorbate glutathione cycle. The increase in activity of enzymes of the ascorbate glutathione cycle has already been demonstrated during fruit ripening (Jimenez et al., 2002). The abundance of carbonic anhydrases in red chromoplasts has been discussed previously as possibly participating in providing CO_2 to Rubisco that is still present at high levels. Another feature of the chloroplast-to-chromoplast transition is related to changes in stress-related, regulatory, and signaling proteins (Fig. 10B). Abiotic stress, redox, and heat shock classes comprise a majority of proteins not changing in abundance but also a number of proteins exhibiting either an increase or a decrease in abundance. The simultaneous changes in redox and abiotic stress proteins are consistent with the role of redox signaling in the response to abiotic stress in plants (Suzuki et al., 2012). The role of some heat shock proteins in promoting color changes during tomato

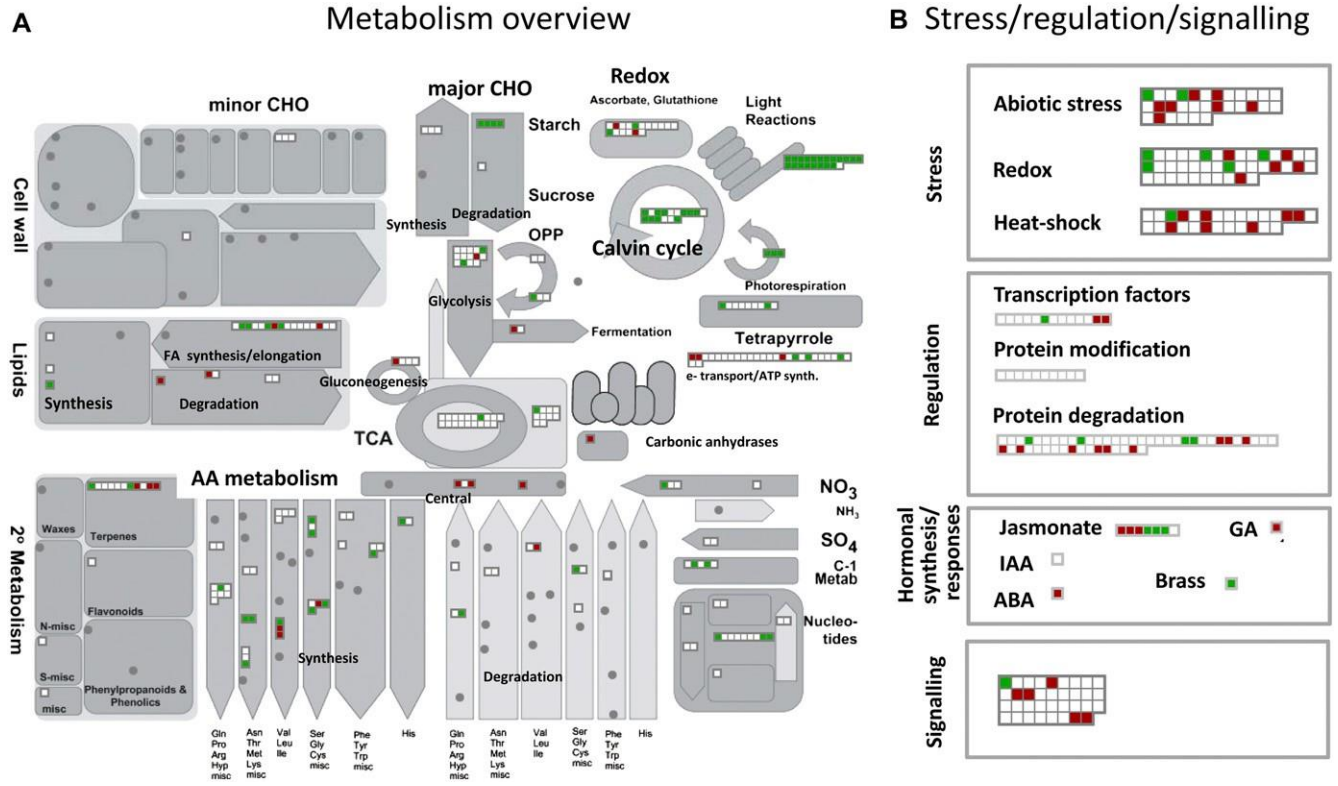


Figure 10. Metabolic overview comparing the protein abundance in MG and R tomato plastids. Red squares represent proteins with decreasing levels while blue squares correspond to proteins with increasing levels. A, Metabolic overview. B, Stress, regulation, and signaling proteins. MapMan software (Thimm et al., 2004; http://gabi.rzpd.de/projects/MapMan/).

fruit ripening has been reported (Neta-Sharir et al., 2005). Moderate redistribution can also be observed in transcription factors, signaling, and proteins involved in protein modification and degradation. Of particular interest are the signaling proteins undergoing an increase in levels. Quantitative changes also occur in proteins involved in the response to hormones and in hormones synthesis. The jasmonate biosynthetic pathway is located in the plastid and several proteins increase in abundance during chromoplastogenesis. This is consistent with the role of jasmonate in the biosynthesis of carotenoid biosynthesis in interaction with ethylene (Fan et al., 1998). Proteins annotated as ethylene related are absent from the MapMan mapping. This could be considered as surprising considering that ethylene plays a major role in the ripening process of climacteric fruit such as the tomato (Pirrello et al., 2009; Pech et al., 2012) and in the synthesis of carotenoids (Bramley, 2002). However, the ethylene biosynthesis and signaling pathways are not located in the plastid and it is expected that the regulation of ethylene-responsive genes occurs mainly at the nucleus. In a microarray analysis of tomato fruit treated by the ethylene antagonist 1-methylcyclopropene, Tiwari and Paliyath (2011) found that at least nine nuclear genes encoding plastidial proteins were up-regulated by ethylene and three down-regulated. Genes involved in the biosynthesis of carotenoids, fatty acids, and jasmonic acid and in gluconeogenesis were up-regulated, while genes involved in starch degradation were down-regulated. Nevertheless, a full inventory of ethylene-responsive genes encoding plastid-localized proteins remains to be performed. In addition, the cross talk between hormones in ripening fruit is not yet well understood at the molecular level.

Loss of the Machinery for the Build Up of Thylakoids and Photosystems

A number of proteins participating in the build up of thylakoids have been encountered that decrease in abundance during the chloroplast-to-chromoplast transition (Fig. 11A; Supplemental Table S4). A nucleoid-binding protein classified in the RNA functional class, MATRIX ATTACHMENT REGION FILAMENT BINDING PROTEIN1 (Solyc03g120230), which is tightly associated with the accumulation of thylakoid membranes (Jeong et al., 2003) decreases in abundance between the green and B stages and is not detectable at the R stage. In addition, the THYLAKOID FORMATION1 (Solyc07g054820) protein, which is involved in thylakoid formation through vesicular trafficking, continuously decreases in abundance. It is thought to

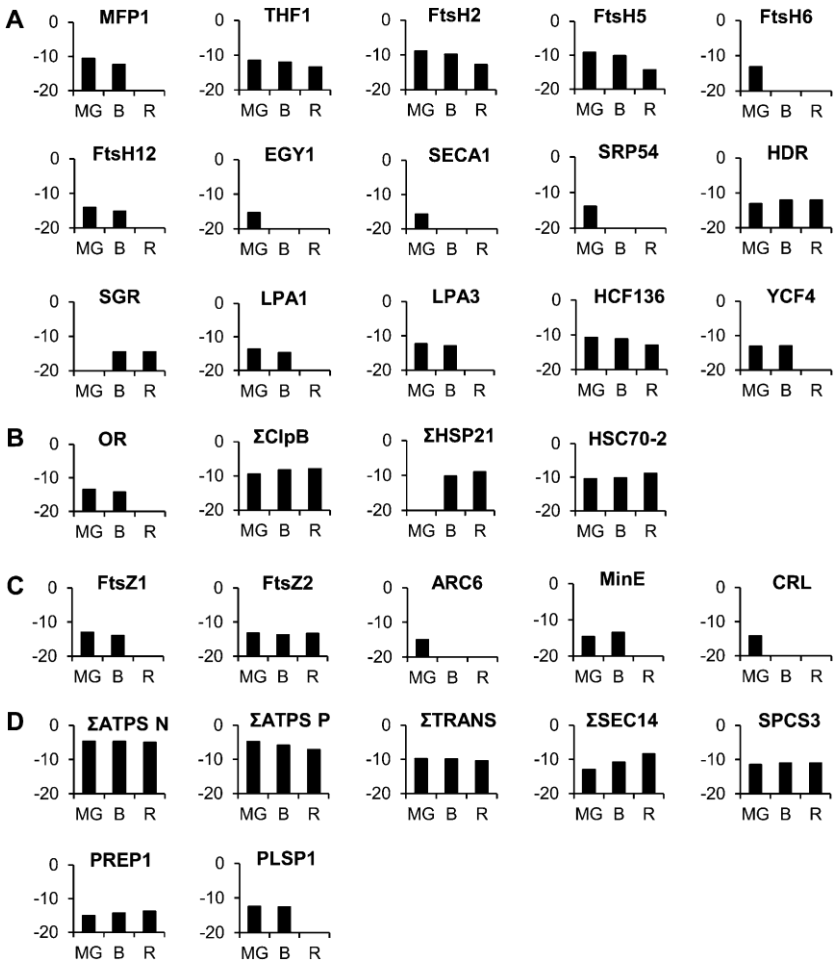


Figure 11. Abundance of proteins (y axis in $\log_2$) involved in structural modifications of plastids, provision of energy, and translocation of precursors during the chloroplast-to-chromoplast transition. A, Proteins involved in the biogenesis of thylakoids and photosystems. B, Proteins involved in plastid differentiation. C, Proteins involved in plastid division. D, Proteins involved in energy and translocation. Protein abundance is expressed as a $\log_2$. The full name of the proteins is indicated in the text and in Supplemental Table S. Abbreviations preceded by S correspond to the sum of several proteins harboring the same function. Individual values are given in Supplemental Table S4.

interact with a plasma membrane G protein to provide a sugar-signaling mechanism necessary for thylakoid formation (Huang et al., 2006). The tomato plastid proteome comprises a number of membrane-bound ATP-dependent metalloproteases of the filamentation temperature-sensitive metalloprotease class (FtsH2: Solyc07g055320; FtsH5: Solyc04g082250; FtsH6: Solyc02g081550; and FtsH12: Solyc02g079000). Members of these proteases are involved in the repair of PSII (Liu et al., 2010a) as well as in the maintenance of the thylakoid structure (Kato et al., 2012). Similarly, another type of ATP-independent metalloprotease, ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW MUTANT1 (EGY1; Solyc10g081470), present in the tomato plastid proteome has been described as required for chloroplast development via its role in thylakoid membrane biogenesis (Chen et al., 2005). The build up of the thylakoids requires the import of proteins within the plastid. A thylakoid Sec translocase subunit (SECA1, Solyc01g080840) is essential for chloroplast biogenesis (Liu et al., 2010b). Interestingly, the FtsH and EGY1 proteins decrease in abundance, mostly between the B and R stages, which correlates well with the dismantling of the thylakoid membranes, while SECA was present only at the MG stage, indicating an early end to the provision of material for thylakoid biogenesis. Recently the lutescent2 mutant of tomato has been identified as mutated for a homolog of EGY1 of Arabidopsis (Barry et al., 2012). The mutation is responsible for an altered chloroplast development and delay in fruit ripening but this not precluded chromoplast differentiation, indicating that chromoplast development does not depend on functional chromoplasts (Barry et al., 2012). A signal recognition particle subunit (SRP54, Solyc09g009940) involved in the integration of the light-harvesting chlorophyll a/b protein into the thylakoid membrane (Li et al., 1995; Rutschow et al., 2008) is present at the MG stage and then absent, giving another indication of the disappearance of photosynthetic protein import during the chloroplast-to-chromoplast transition.

Consistent with the decrease in the chlorophyll biosynthetic branch, an increase is observed of the STAY-GREEN (Solyc08g080090) protein that regulates chlorophyll degradation by modulating pheophorbide a oxygenase activity (Ren et al., 2007). Another protein, HYDROXYMETHYLBUTENYL DIPHOSPHATE REDUCTASE (Solyc01g109300) produces MEP-derived precursors for plastid carotene biosynthesis. It increases slightly during the three stages

that is consistent with the accumulation of carotenoids and in agreement with the up-regulation of the HYDROXYMETHYLBUTENYL DIPHOSPHATE REDUCTASE genes during tomato fruit ripening (Botella-Pavía et al., 2004). In addition, there is also an abrupt decrease, between the B and R stages, of two low PSII accumulation proteins (LPA1: Solyc09g074880 and LPA3: Solyc06g068480) involved in the assembly of PSII (Peng et al., 2006; Cai et al., 2010). The two proteins have been clearly identified by Peng et al. (2006) and Cai et al. (2010) but still annotated as unknown gene in The Arabidopsis Information Resource and Solyc databases. Another protein necessary for the assembly of PSII high chlorophyll fluorescence (HCF136, Solyc02g014150; Plücken et al., 2002) decreases continuously. A plastid genome-encoded protein, YCF4 (Solyc01g007360) belonging to the hypothetical chloroplast reading frame family (YCF) decreases strongly between the B and R stages in parallel with the late disassembly of PSI. The Ycf4 protein participates in the assembly of PSI in the alga Chlamydomonas reinhardtii (Onishi and Takahashi, 2009) and in higher plants (Krech et al., 2012). However, unlike its role in algae, in higher plants it is not essential. The knockdown of the YCF4 gene in tobacco (Nicotiana tabacum) results in an only partial reduction of the amount of PSI complex, indicating that other proteins can replace YCF4 (Krech et al., 2012). Similarly to the decline in the YCF4 protein during the transition from chloroplast to chromoplast observed here in tomato fruit, YCF4 declines continuously in old leaves of tobacco concomitantly with the decrease of photosynthetic activity (Krech et al., 2012).

Several Elements of Plastid Differentiation Correlate with Chromoplast Formation

Interestingly, the tomato fruit plastid proteome contains a number of proteins known to participate in the differentiation of plastids (Fig. 11B; Supplemental Table S4). One of them is a dnaj-like chaperone (Solyc03g093830) and corresponds to the product of the Or gene that controls chromoplast differentiation and carotenoid accumulation (Li and Van Eck, 2007). The Or protein is more abundant at the B than at the green stage and was not detected at the R stage when carotenoids are at their maximum level.

Some ATP-dependent casein lytic proteinases (Clp) located in the stroma are described as participating in chloroplast development (Lee et al., 2007). Six of them classified in the stress-related proteins (ClpB: Solyc06g082560, Solyc03g115230, Solyc02g088610, Solyc06g011400, Solyc06g011370, and Solyc06g011380) are encountered in the tomato plastids. Summing the abundance of the six proteins remains essentially constant, suggesting a sustained function during chromoplast differentiation. Also interesting is the absence at the G stage and the sharp accumulation at the B and R stages of two HEAT SHOCK PROTEIN21 (HSP21) heat shock proteins (Solyc03g082420 and Solyc05g014280) that have been reported to be involved in the promotion of color changes during tomato fruit maturation (Neta-Sharir et al., 2005). Western blots show the presence of HSP21 proteins at very low level at the MG stage and a sudden and large increase at the B stage (Fig. 6). Except for the presence of small amounts at the MG stage in western blots, these data are in agreement with the proteomic analysis. Another heat shock protein, HSC70-2 (Solyc11g020040), highly expressed in the tomato plastids, increases continuously. It has been described as essential for plant development in Arabidopsis (Su and Li, 2008). Its role in chromoplast differentiation would deserve elucidation.

Loss of the Plastid Division Machinery

Many proteins involved in the division of plastids have been encountered in the tomato plastid proteome (Fig. 11C; Supplemental Table S4). Two nuclear-encoded forms of filamenting temperature-sensitive Z mutants (FtsZ1, Solyc07g065050 and FtsZ2, Solyc09g009430) play a major role in the initiation and progression of plastid division in plant cells (McAndrew et al., 2008). They are both present at MG and B stages, but FtsZ1 was absent at the R stage while FtsZ2 remained stable at all three stages. An accumulation and replication of chloroplasts protein (ARC6, Solyc04g081070) has been characterized in a mutant that has only one or two chloroplast per cell instead of ~100 in wild-type cells (Vitha et al., 2003). The mutant exhibits abnormal localization of the two key plastid division proteins FtsZ1 and FtsZ2, indicating that ARC6 promotes FtsZ filament formation in the chloroplast (Aldridge et al., 2005). ARC6 was encountered at the MG stage only. The MinE protein (Solyc05g012710), which supports and maintains FtsZ filament formation (Maple and Møller, 2007) and is required for correct plastid division in Arabidopsis (Maple et al., 2002), disappears between the MG and B stages. Similarly, the crumpled leaf protein (CRL, Solyc06g068760), also described as important for the division of plastids, was found only at the MG stage. Asano et al. (2004) showed that cells of the CRL mutant contained a reduced number of plastids. The disappearance or strong decrease of the majority of proteins involved in plastid division mentioned above during the differentiation of chromoplasts indicates that plastid division ceases. This is in agreement with our previous observation that no plastid division occurred in ripening tomatoes since all preexisting chloroplasts were differentiating into chromoplasts (Egea et al., 2011). These data provide target proteins potentially responsible for the cessation of plastid division.

Proteins Involved in Energy Provision and Translocation Activities

ATP synthase is an important enzyme that provides energy for the cell to use through the synthesis of ATP. Thirteen nuclear-encoded ATP synthase units were quantified in the tomato plastid proteome (Supplemental Table S). The summation of the abundance of all these

ATP synthase units was stable during chromoplast differentiation (Fig. 11D). On the other hand, six ATP synthase units encoded by the plastid genome were quantified (Supplemental Table S4) and slightly decreased during tomato fruit ripening (Fig. 11D). These data indicate that the machinery for the provision of energy stays very present throughout the differentiation process. Plastids can import cytosolic Glc-6-P via the Glc-6-P/phosphate translocator (GPT, Solyc07g064270). Glc-6-P is used either for the synthesis of starch and fatty acids or is fed into the plastidic OPP (Emes and Neuhaus, 1997). Niewiadomski et al. (2005) have shown that the loss of GPT1 function results in a disruption of the oxidative pentose phosphate cycle and affects fatty acid biosynthesis. Another translocator, phosphoenolpyruvate phosphate translocator (Solyc03g112870), has been identified that delivers the energy-rich glycolytic intermediate phosphoenolpyruvate into the plastids (Fischer et al., 1997). In addition, a triose phosphate/phosphate translocator (Solyc10g008980) has also been encountered that participates in Suc biosynthesis (Cho et al., 2012). The sum of the three translocators decreases very lightly during plastid transition (Fig. 11), indicating that the machinery for provision of energy and precursors remained in place to allow the synthesis of fatty acids and Suc within the plastid.

Elements for the direct import of lipids into the plastid are also present (Fig. 11D; Supplemental Table S4). Two proteins of the SEC translocase system are highly expressed and increase continuously in abundance (SEC14, Solyc11g051160, Solyc06g064940). They are the homologs of the yeast (Saccharomyces cerevisiae) SFH5 phosphatidylinositol transfer protein (Yakir-Tamang and Gerst, 2009) and could be involved in Golgi vesicle transport of phosphoinositides in plant plastids. The transport of lipids to the plastids via vesicles derived from the endoplasmic reticulum membrane and fused with Golgi membranes is more than a hypothesis (Andersson and Dörmann, 2009; Benning, 2009). Elements of the protein import machinery were also encountered. One signal peptidase complex subunit (SPCS3, Solyc01g098780) and one presequence protease (PREP1, Solyc01g108600) remained roughly stable, indicating that elements of the machinery for import of proteins remain present during chromoplast differentiation, while the synthesis of protein by the plastid translational machinery strongly declines, as mentioned above. Another signal peptidase, plastidic signal peptidase (PLSP1, Solyc12g007120) involved in thylakoid development through its involvement in processing the Toc75 envelope protein and 0E33 thylakoid luminal protein (Shipman-Roston et al., 2010) was absent at the R stage, which is consistent with thylakoid dismantling.

CONCLUSION

High-throughput technologies have been used in the recent years for elucidating the mechanisms of fruit ripening, such as transcriptomics (Alba et al., 2004), metabolomics (Schauer et al., 2006; Deborde et al., 2009; Moing et al., 2011), proteomics (Rocco et al., 2006; Faurobert et al., 2007; Palma et al., 2011), and more recently, a combination of all of them (Osorio et al., 2011). However these technologies have been scarcely used in the study of subcellular organelles such as chromoplasts. In this article, we used high-throughput proteomics to make an inventory of the proteins present at different stages of plastid differentiation in ripening tomato fruit. This inventory enriches the knowledge of the plant plastid proteome as reported in plastid databases. In addition, it provides an in-depth description of the changes occurring in the plastidial proteome during chloroplast-to-chromopast differentiation. The mechanisms governing the differentiation of plastids such as the conversion of proplastids to chloroplasts or chloroplasts to chromoplasts have received little attention and are barely mentioned in recent reviews on plastid proteomics (Armbruster et al., 2011; van Wijk and Baginsky, 2011). The pattern of changes in protein abundance reported here is in agreement with proteomic data generated in whole fruit by others (Rocco et al., 2006; Faurobert et al., 2007). The western blots of six proteins representative of several metabolic or regulatory pathways give a pattern of changes that is similar to proteomic analysis. Therefore, these data confirm the reliability of the tandem mass spectrometry (MS/MS) analysis and the spectral counting quantification procedure carried out in this study. In addition, the evolutions of protein abundance are in agreement with the most important metabolic changes described in the literature such as chlorophyll degradation, loss of photosynthetic activity, dismantling of thylakoid membranes, and increase in the synthesis of lycopene (Egea et al., 2010). Previous studies had sequentially described individual metabolisms or even specific steps of metabolism. In this work a global and extensive quantitative picture is given of the major shifts occurring during the differentiation of chromoplasts that integrate both the strong decrease in proteins of the light reactions and major CHO metabolism and the increase in abundance of proteins involved in carotenoid biosynthesis. A remarkable finding not described so far is the stability of proteins of the TCA cycle, glycolysis, and the high level of electron transport/ATP synthesis, indicating that the machinery providing energy to the plastid is largely conserved. Particularly noticeable is also the increase in a number of proteins of the ascorbate glutathione cycle, abiotic stress, redox, and heat shock classes, indicating that the transition seems to be governed at least partly by a redox-signaling pathway causing a stress-related response. Strong redistribution can also be observed in transcription factors, signaling, proteins involved in protein-modification/degradation, and in proteins involved in the response to and synthesis of hormones. This indicates that chromoplastogenesis involves profound changes in signaling events whose details and interactions remain to be elucidated. The demonstration that chromoplasts can develop from altered chloroplasts in lutescent2 tomato fruit mutants (Barry et al., 2012)

indicates that the differentiation of chromoplasts and the associated signaling events do not depend on functional chloroplasts. However, although with some delay, chromoplast formation always accompanies fruit ripening.

Among the most important events of chromoplast differentiation the dismantling of thylakoids have been well described in the literature by electron microscopy. This study brings novel information on the target proteins participating in the loss of the machinery for the build up of thylakoids and for the assembly of photosystems. For instance, proteins involved in thylakoid formation through vesicular trafficking, in the provision of material for thylakoid biosynthesis, and in the assembly of photosystems undergo a strong decrease in abundance to be essentially absent in red chromoplasts. In agreement with the loss of chlorophylls, the stay-green protein that stimulates chlorophyll degradation increases in abundance. Proteins of the plastid division machinery have been identified that disappear during the differentiation of chromoplasts. Their absence in fully differentiated chromoplasts can therefore be considered as responsible for the cessation of plastid division. An unexpected finding is that chromoplast differentiation is accompanied with the maintenance of the major elements providing energy and metabolites to the plastid such as ATP synthase, hexose, and triose phosphate translocators, as well as lipid import elements. The translation machinery of the plastid probably undergoes a great loss of efficiency with the strong decrease in abundance of ribosomal proteins of both the 30S and 50S complexes. However, several elements of the protein import machinery remain at a high level, suggesting that nuclear-encoded proteins continue to be transferred at a high rate till the last stage of chromoplast differentiation.

Our results complement recent studies on chloroplast-to-chromoplast differentiation, showing that chromoplast gene expression largely serves the production of a single protein, ACCD (Kahlau and Bock, 2008). We now demonstrate that this occurs early during fruit development while the fruit is unable to ripen autonomously and well before any visible transition from chloroplast to chromoplast. We conclude the early accumulation of the ACCD protein that is involved in fatty acid biosynthesis is a prerequisite for chromoplast differentiation by providing a storage matrix for the accumulation of carotenoids. The final steps of differentiation during which the metabolic shifts occur are associated with the initiation of the ripening process by the plant hormone ethylene.

These data demonstrate that the shifts in metabolism are preceded by the increase of the ACCD protein that could account for the generation of a cartenoids storage matrix. They also show that the metabolic changes are associated with a high abundance of proteins involved in providing energy and import of metabolites. In addition, regulatory proteins have been identified that are potentially responsible for the overall differentiation process as well as for individual differentiation events such as the dismantling of thylakoids and photosystems and cessation of division.

MATERIALS AND METHODS

Plant Material

Tomato (Solanum lycopersicum 'MicroTom') plants were cultivated under standard greenhouse conditions. Fruit were collected at three stages of ripening after careful selection: (1) MG fruit corresponds to fruit having reached full size and able to ripen autonomously. They produce very low amounts of ethylene but respond to ethylene in terms of ripening. MG fruit were selected by the presence of gel in the locules and an a*/b* chromatic ratio between 20.32 and 20.38 measured by the Minolta chromameter; (2) B + 2 corresponds to fruit harvested 2 d after the B stage. B is characterized by a change in color from green to pale orange over about 30% of the surface. At B + 2, the fruit reach peak ethylene production; (3) R fruit has the whole surface colored red and were harvested 10 d after B.

Plastid Isolation from Fruit at Various Stages of Ripening and Fractionation of Proteins

Plastids were isolated from the fruit pericarp at three stages of ripening according to the procedure described in Egea et al. (2011). After separation on Suc gradient, plastid fractions were analyzed by fluorescence confocal microscopy associated with spectrophotometric analysis. MG plastids were characterized by the almost exclusive presence of chlorophylls, characteristic of chloroplasts; B plastids corresponding to intermediate forms of plastids were selected in a band of gradient containing reduced amounts of chlorophylls and substantial amounts of carotenoids; R plastids contained almost exclusively carotenoids, typical of chromoplasts. The plastid bands collected were washed twice with extraction buffer (250 mM HEPES, 330 mM sorbitol, 0.1 mM EDTA, 5 mM b-mercaptoethanol, pH 7.6). For the proteomic analysis plastids were resuspended in 1 M HEPES buffer (pH 7.6), 2 mM dithiothreitol (DTT), and kept at 220°C until protein precipitation. After storage at 220°C plastids were broken by osmotic shock by resuspension in 1 M HEPES buffer (pH 7.6) complemented with 2 mM DTT, followed by freeze/thawing and homogenization in a Potter-Elvehjem tissue grinder. Proteins of each fraction were precipitated with methanol/chloroform (3:8 v/v) according to Seigneurin-Berny et al. (1999) with some modifications. The resulting protein pellet was dissolved in 2% SDS, 62.5 mM Tris-HCl buffer (pH 6.8). Protein concentrations were determined using the bicinchoninic acid method (Smith et al., 1985). Finally the proteins were reduced with DTT (20 mM) and alkylated with chloroacetamide (60 mM) before solubilization in Laemmli buffer and SDS-PAGE (12% acrylamide) separation. Each lane of the one-dimensional SDS-PAGE gel was loaded with 70 mg of proteins originating from the three types of plastids. After electrophoresis, proteins were stained with PageBlue protein staining solution (Fermentas). Three independent biological replicates of SDS-PAGE gel were carried out for each plastid development stage (Fig. 1).

Western-Blot Analysis

Western-blot analysis was performed using polyclonal antibodies at appropriate dilution against chloroplastic RBCL (53 kD, at 1:50,000 dilution), PSI PSAD (23 kD, at 1:25,000 dilution), PSII PSBA/D1 protein (39 kD, at 1:50,000 dilution), HSP21 (26 kD, at 1:30,000 dilution), and LOXC (102 kD, at 1:50,000 dilution) from Agrisera. ACCD (58 kD) antibodies were purchased from the Plant Antibody facility of the Ohio State University and used at 1:25,000 and 1:15,000 dilution in Figure 6 and Supplemental Figure S2, respectively. Extraction of proteins, separation by SDS-PAGE, and detection by antibodies were performed as described in Barsan et al. (2010). Proteins were extracted from partially purified plastids recovered at the step before separation on Suc gradient as described in Egea et al. (2011).

Trypsin Digestion and Liquid Chromatography MS/MS Analyses of Gel Segments

The protocol was the same as in Barsan et al. (2010) except that each lane of the gel was cut into 10 slices.

Protein Identification and Quantification by Spectral Counting

Data were analyzed using Xcalibur software (version 2.1.0, Thermo Fisher Scientific) and MS/MS centroid peak lists were generated using the

extract_msn.exe executable (Thermo Fisher Scientific) integrated into the Mascot Daemon software (Mascot version 2.3.2, Matrix Sciences). The following parameters were set to create peak lists: parent ions in the mass range 400 to 4,500, no grouping of MS/MS scans, and threshold at 1,000. A peaklist was created for each fraction (i.e. each gel slice) analyzed and individual Mascot searches were performed for each fraction. Data were searched against iTAG2.3 proteome sequence (ftp://ftp.solgenomics.net/tomato_genome/annotation/ITAG2.3_release/ITAG2.3_proteins.fasta) predicted from the tomato genome sequence by the International Tomato Genome Annotation (iTAG) team and against the proteome sequences predicted from the chloroplastic genome (accession no. AM087200). Mass tolerances in mass spectrometry and MS/MS were set to 5 ppm and 0.8 D, respectively, and the instrument setting was specified as ESI Trap. Trypsin was designated as the protease (specificity set for cleavage after Lys or Arg) and one missing cleavage was allowed. Carbamido methylation of Cys was fixed and oxidation of Met was searched as variable modification. Mascot results were parsed with the homemade and developed software MFPaQ version 4.0 (Mascot File Parsing and Quantification; Bouyssié et al., 2007). To evaluate false positive rates, all the initial database searches were performed using the decoy option of Mascot, i.e. the data were searched against a combined database containing the real specified protein sequences (iTAG2.3 tomato proteome sequence) and the corresponding reversed protein sequences (decoy database). MFPaQ used the same criteria to validate decoy and target hits, calculated the false discovery rate (FDR; FDR = number of validated decoy hits/[number of validated target hits + number of validated decoy hits] 3 100) for each gel slice analyzed, and made the average of FDR for all slices belonging to the same gel lane (i.e.to the same sample). FDRs were below 1.6%. From all the validated result files corresponding to the fractions of a one-dimensional gel lane, MFPaQ was used to generate a unique nonredundant list of proteins that were identified and characterized by homology-based comparisons with iTA1G2.3 tomato proteome sequence. Output files from Mascot searches were uploaded with MFPaQ for spectral counting. The total number of spectra corresponding to each identified protein was extracted from each lane of analyses. The filtering parameters were P values - 0.05, more than seven amino acids in the peptides and more than one peptide encountered. Among all strategies used for quantitative proteomics (Thelen and Peck, 2007; van Wijk and Baginsky, 2011) we followed a label-free option of spectral counting (Liu et al., 2004; Booth et al., 2011) described in Bouyssié et al. (2007). The spectral-count-based label free has been reported as allowing quantification of protein abundance with similar efficiency to isotope labeling (Zhu et al., 2009). This methodology had been applied in a number of other studies related to the plastid proteome (Bräutigam et al., 2008; Ferro et al., 2010; Demartini et al., 2011).

Comparison with Existing Databases, Targeting Predictions, Functional Classification, and Curation

Protein descriptions were performed using annotations associated with each protein entry and through homology-based comparisons with the TAIR9 protein database (http://www.arabidopsis.org/) using BasicLocal Alignment Search Tool BLASTX (Altschul et al., 1990) with an e-value cutoff of 1e-5 to avoid false positives, and linked. MapMan Bins were used for functional assignments (http://gabi.rzpd.de/projects/MapMan/). The protein list was compared with five plastidial databases either specific to plastids (AT-CHLORO: Ferro et al., 2010; http://www.grenoble.prabi.fr/proteome/grenoble-plant-proteomics/; plprot: Kleffmann et al., 2006; http://www.plprot.ethz.ch) or general databases comprising subcellular subsets (PPDB: Sun et al., 2009; http://ppdb.tc.cornell.edu; SUBA: Heazlewood et al., 2007; http://www.suba.bcs.uwa.edu.au; Uniprot: The Uniprot Consortium, 2010: http://www.uniprot.org). Predictions of subcellular localization were undertaken using three predictors (TargetP: Emanuelsson et al., 2000; http://www.cbs.dtu.dk/services/TargetP/; iPSORT: Bannai et al., 2002; http://hypothesiscreator.net/iPSORT/; Predotar: Small et al., 2004; http://genoplante-info.infobiogen.fr/predotar/). Predictions were made on the basis of tomato proteins when harboring an N-terminal sequence. A curation procedure was used to select, in the protein data set, those having confirmed plastid location. Proteins were retained in the final dataset if they meet with at least one of the two following criteria: (1) presence in at least two of the five databases mentioned above (AT-CHLORO, plprot, PPDB, SUBA, and Uniprot) and (2) predicted by at least one of the three predictors (TargetP, iPSORT, and Predotar). Proteins predicted to be encoded by the plastid genome were all retained. This resulted in the cataloging of 1,932 proteins in Supplemental Table S1.

Normalization and Differential Abundance Analysis

The raw data arising from the LTQ-Orbitrap mass spectrometer analysis (1,932 proteins listed in Supplemental Table S1 were submitted to a normalization process that consisted in standardizing the MS/MS counts by the length of the corresponding protein, and by an integral normalization, as described by Paoletti et al., 2006). The sum of abundances in all samples was set to 1 so as to eliminate the effect of the size of the total MS/MS counts in the different samples. Then, the Probabilistic Quotient Normalization method (Dieterle et al., 2006) was applied by taking a reference sample at random among the replicates of the MG stage. This allowed the calculation of the quotients of protein abundances (as $\log_2$ ratios) in each sample and hence the median of the quotients. By dividing the abundance of proteins by the corresponding median, the effect of the difference in proteome size between samples was eliminated. The normalization process is now widespread and considered as a standard way to preprocess data in metabolomics and proteomics (Torgrip et al., 2008; Issaq et al., 2009; Kato et al., 2011).

The differential abundance analysis was carried out with the Anapuce R package in the R environment (http://www.R-project.org/) and calculated using a paired t test on normalized data. An estimation of the variance for each protein observation was performed using the mixed model of Delmar et al. (2005a, 2005b) implemented in the anapuce R package and consisting in the estimation of several groups of proteins with the same variance. Finally, a classical FDR procedure was applied to correct for multiple comparison tests according to the procedure of Benjamini and Hachberg (1995) implemented in the anapuce R package. Normalization and statistical analysis led to the cataloging of 1,529 proteins considered as quantifiable out of the initial 1,932 proteins (Supplemental Table S2). Protein abundance values (expressed as $\log_2$) were retained only when a given protein had been detected at least twice in a given stage. Supplemental Table S2 comprises the $\log_2$-abundance values, the $\log_2$ ratios, and the P value of the t test for each protein. The trends of changes in abundance between stages were calculated with a 5% significance level and referred to as 0 for no change, +1 for increasing, and 21 for decreasing.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison of the percentage of proteins classified into MapMan functional classes between the tomato fruit chloroplast proteome described in this article (white bars) and the Arabidopsis chloroplast proteome described in the AT-CHLORO database (Ferro et al., 2010; gray bars) and in Zybailov et al. (2008; black bars).

Supplemental Figure S2. Western-blot analysis of the ACCD protein in plastids isolated at four stages of fruit development: IG (Immature-Green).

Supplemental Table S1. Inventory of the 1,932 proteins representing the compilation of the curated list of proteins encountered in the three replicates of the tomato plastids at three stages of development.

Supplemental Table S2. Inventory of 1,529 proteins quantified from the curated list.

Supplemental Table S3. List of proteins in the subplastidial compartments: envelope, stroma, thylakoids, and plastoglobules.

Supplemental Table S4. List of proteins involved in the build up of thylakoids and photosystems, plastid differentiation, plastid division, and energy and translocation.

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Chapter III-Effects of inhibiting protein translation in the plastid on fruits ripening and expression of nuclear genes

Abstract

Several fruit ripening pathways occur in the chromoplast. The most obvious of them is the changes in color from green to red corresponding to the loss of chlorophylls and accumulation of carotenoids. Most of the proteins involved in the ripening process are encoded by the nuclear genome and are imported into the plastid. The plastid genome encodes approximately only 80 genes. Therefore, among the 3500-4000 proteins which are estimated to be present in the plastid, the large majority is encoded by nuclear genes. The coordination between nuclear and plastidial gene expression has become an important biological question. The search of signals participating in this interaction has been undertaken for the photosynthetic process in chloroplasts, but not for carotenogenesis in chromoplasts. This chapter is a preliminary attempt to address this question. In order to check whether plastid genes expression has a feedback on gene expression in the nucleus, we have inhibited protein synthesis in the plastid using the antibiotic lincomycin. We have observed a delay in coloration and carotenoid accumulation in lincomycin-treated fruit. The expression of a number of nuclear genes involved in carotenoid and ethylene biosynthesis has been assessed as well as the expression of transcription factors known to regulate the fruit ripening process. However, although the expression of these genes was affected by the lincomycin treatment, no clear relationship with the delay in carotenoid accumulation could be established.

Introduction

One of the most visible aspects of the fruit ripening process in tomato is the change in color from dark green to yellow and red. It corresponds to the loss of chlorophylls and the accumulation of carotenoids inside the plastids during the transition from chloroplasts to chromoplasts. Plastids are plant-specific, semiautonomous organelles that have important metabolic functions. In higher plants, plastids differentiate from different

subtypes in response to environmental conditions and over several developmental stages. Each differentiated form of plastids plays a specific role in maintaining a variety of metabolic and physiological functions as a component of plant cells or tissues. The type of plastids depends on their function in the cell in which they reside (Egea et al., 2010; Bian et al., 2011). The most characterized form of plastids found in plants are chloroplasts, which are present in green tissues and are involved in photosynthesis (Murakami, 1965). Chromoplasts occur in a limited number of organs, such as fruits and floral petals, or root tissues of carrot and are engaged in the synthesis and storage of pigments (Camara et al., 1995). Leucoplasts are colorless plastids mainly existing in root tissues and are the site of several reactions such as terpene biosynthesis (Gupta et al., 2011). Amyloplasts are non-pigmented organelles found in some plant cells that are responsible for the synthesis and storage of starch granules (Enami et al., 2011; Stanga et al., 2011). Etioplasts are chloroplasts that have not been exposed to light, they are usually found in flowering plants grown in the dark (Wellburn and Wellburn, 1971, 1971).

It is considered that plastids evolved from cyanobacteria that were incorporated into a eukaryotic host cell (Dyall et al., 2004). During the process of evolution, the transfer of genes from the prokaryotic endosymbiont to the nucleus of the host cell provided an opportunity for increased control of the endosymbiont and its biological functions by the host cell. The size of the plastid genome in land plants was reduced to about 80 genes or less. Therefore, plastid biogenesis is dependent on the expression of nuclear-encoded plastid proteins and their posttranslational import into plastids (Inaba and Schnell, 2008; Li and Chiu, 2010).

Communication between the plastids and the nucleus is bidirectional. Anterograde signaling corresponds to the convey from the nucleus to the plastids of signals that stimulate gene expression in the plastid (Pesaresi et al., 2007; Kleine et al., 2009). In contrast, retrograde signaling correspond to the regulation of nuclear gene expression by signals emitted by the plastid (Nott et al., 2006; Pogson et al., 2008; Inaba, 2010; Pfannschmidt, 2010). These signals reflect both the developmental and functional state of the plastid. A recent review paper gives more details of the plastid-to-nucleus

communication (Barajas-Lopez et al., 2012). A number of mutants have been characterized that are affected the retrograde signaling.

The phenotype of genome uncoupled (GUN) mutants varies from pale yellowish to undistinguishable from wild-type (Surpin et al., 2002). Gun genes are involved in modulating magnesium-protoporphyrin IX (Mg-proto IX) levels (Vinti et al., 2000; Larkin et al., 2003; Strand et al., 2003) which were considered as a factor involved in the plastid to nucleus signal (Mochizuki et al., 2001). Loss of function of gun mutants led to increased expression of photosynthesis-associated nuclear genes (PhANGs) when chloroplast development was blocked by norflurazon. One exception, GUN1, is unique since it exhibited derepression of LHCB genes after treated with norflurazon or lincomycin (Mochizuki et al., 2001; Susek et al., 1993; Dietzel at al., 2009). GUN1 is insensitive to accumulation of Mg-proto IX which is considered to be a signal factor. Gun1 gene plays a role in the Mg-proto IX pathway, but it does not seem to be involved in chlorophyll biosynthesis, which indicates that it most likely acts downstream of Mg-proto IX accumulation (Koussevitzky et al., 2007). The others gun mutants have the similar function for increasing plastid photosynthesis-related genes expression treated by norflurazon or lincomycin. Gun2 and gun3 mutant alleles were encoding haem oxygenase and phytochromobiline synthase respectively. With absence of the two enzymes, plastid accumulated heme leads to negative feedback regulation of chlorophyll biosynthesis (Mochizuki et al., 2001). Gun4 and gun5 were found to be directly involved in the chelation of magnesium into protoporphyrin IX. Gun 4 was found to encode an activator of Mg-chelatase and gun5 was encoded a subunit of Mg-chelatase. Recently, a new mutant, gun6-1D, with a similar phenotype to the gun2-gun5 has been reported (Woodson et al., 2011). In the gun6-1D mutant, a 3-fold increasing of the ferrochelatase 1 and the activity was found. Subsequently, heme was increased and expression of PhANGs was up-regulated. These data suggest a model that heme may be used as a retrograde signal to coordinate PhANG expression with chloroplast development (Woodson et al., 2011).

A mutant screened from norflurazon treated Arabidopsis seedlings called happy on norflurazon (HON) down-regulated the expression of Lhcb genes more indirectly prior to

initiation of plastid signaling. HON mutations disturb plastid protein homeostasis, thereby activating plastid signaling (Saini et al., 2011). Kakizaki et al. (2009) showed a mutant lack a most abundance protein import receptor atToc 159. With no proteins import in to the plastid, the expression of photosynthesis-related nuclear genes was down-regulated. They suggested a signal that repress the photosynthesis-genes expression through repress the expression of AtGLK instead of Mg-proto IX accumulation.

The present chapter is intended at determining whether a retrograde signaling exist between the plastid and the nucleus for inducing the transition between chloroplast to chromoplast and the associated metabolic changes. In order to address the question we have infiltrated in the fruit a specific inhibitor of the translation of proteins in the plastid, lincomycin. It has already been used in vivo to study its influence on chromoplast tubules formation (Emter et al., 1990), chloroplast ultra-structure elements (Kryloy and Masikevich, 1996), chlorophyll-protein complex formation (Guseinova et al., 2001), expression of nuclear photosynthetic genes (Sullivan and Gray, 1999, 2002) and acclimation of the photosynthetic machinery (Tanaka et al., 2000). In our work, we have studied the effects of lincomycin on fruit ripening and on the expression of ripening-related genes including carotenoid and ethylene biosynthesis genes and transcription factors controlling the ripening process.

Since the chloroplast-to-chromoplast transition is primarily associated with the accumulation of carotenoids, we have used carotenoid biosynthesis genes as targets for detecting the effects of lincomycin. Upstream in the pathway is the plastid-localized methylerythritol (MEP) pathway that provides precursors, IPP, DMAPP and GGPP for the synthesis of carotenoids (Phillips et al., 2008). Overexpression of hydroxymethylbutenyl diphoshate reductase (HDR) results in an increased production of carotenoids in light-grown Arabidopsis seedlings (Estévez et al., 2001; Botella-Pavía et al., 2004; Carretero-Paulet et al., 2006; Flores-Perez et al., 2008). GGPP synthase (GGPS) generates the 20-carbon GGPP molecule, which serves as the immediate precursor for carotenoids. The first committed step in plant carotenoid biosynthesis is the synthesis of phytoene from GGPP and IPP. Phytoene synthase (PSY) involved in this step. Tomato fruits contains two isoforms of the PSY gene, PSY1 and PSY2. The PSY1 encodes the

fruit-ripening-specific isoform, while PSY2 has no role in carotenogenesis in ripening fruits. Phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), carotene isomerase (CRTISO), plastid terminal oxidase (PTOX) are involved in lycopene biosynthesis. The enzymes involved in the degradation of the lycopene are lycopene β-cyclase (LCY-B), lycopene ε-cyclase (LCY-E) and β-carotene hydroxylase 2 (CRTR B). The products of these enzymes are different carotene isomers.

Among other ripening-related genes, we have also studied genes involved in the biosynthesis of ethylene. In the ethylene biosynthesis pathway, 1-aminocyclopropane-1-carboxylic acid (ACC) is catalyzed by ACC synthase (ACS), which is the rate-limited step in ethylene biosynthesis. ACC oxidase (ACO) then converts ACC to ethylene (Wang et al., 2002). ACS is encoded by a multigene family. Barry et al. (2000) showed that transcripts of ACS1A is decrease sharply from breaker stage fruits to ripening and Nakatsuka et al. (1998) indicated that ACO1 increasing transcripts during ripening.

Beside genes involved in the biosynthesis of carotenoids and ethylene, some other genes corresponding to transcription factors controlling the ripening process have been taken into account: the tomato MADS-Box Transcription Factor ripening inhibitor (RIN) mutant is a loss-of-function mutant (Zhu et al., 2007). The color change, softening and aroma production of this mutant are inhibited and the fruit fails to ripen. Another mutation named colorless non-ripening (CNR) results in mature fruits with colorless pericarp tissue and an excessive loss of cell adhesion (Thompson et al., 1999). The two mutants show deficient accumulation of pigments during tomato fruit ripening.

Few genes controlling the differentiation of chromoplasts have been discovered. For instance, the Orange (*Or*) gene has been identified in a mutation of cauliflower accumulating high levels of β-carotene in various tissues normally devoid of carotenoids. Or is most likely not a loss-of-function mutant of the wild-type *Or* gene with no carotenoid accumulation in RNAi mutant (Lu et al., 2006). Also, a plastid fusion and/or translation factor (PFTF) has been characterized. It has been involved in chromoplast differentiation in red pepper (Hugueney et al., 1995).

The objective of the present work was to make a preliminary understanding of the signaling between chromoplast and nucleus. We used lincomycin as the inhibitor of

plastidial translation to check whether the expression of nucleus genes were affected by the blocking. Tomatoes were injected with lincomycin and some color and ripening related genes was analyzed by qPCR. These results will enhance the understanding of the communication between plastid and nucleus and provide a preliminary work of signaling between chromoplast and nucleus.

Material and Methods

Tomato Material and growth conditions

Tomato (*Solanum lycopersicum* cv Micro Tom) plants were grown under standard greenhouse conditions. The conditions for the culture chamber room are as follows: 14-h-day/10-h-night cycle, 25/20℃ day/night temperature, 80% humidity, 250 mmol $m^{-2}s^{-1}$ intense luminosity. Harvest at breaker stage which is characterized by a change in color from green to pale orange at about 30% of the surface.

Tomato fruits Injected with lincomycin solution

Tomato fruits were collected from green house at breaker stage, all the fruits washed twice with distilled water and dry in the air. Injection was done as indicated by (Orzaea et al., 2006) with modification. A 1 ml sterile hypodermic syringe with a 0.45 ×12 mm needle (TERUMO) was used for infiltration. Needle was introduced 4 to 6 mm in depth into the fruits through the stylar apex, and the infiltrated solution (sorbitol 3% and MES 10 mM pH 5.6) with lincomycin (2mM) was gently injected into the fruits. Control fruits inject with only infiltrated solution. The total volume of solution injected varied to the size of the fruits with a volume approximately 300 μL. Once the entire fruit surface has been infiltrated, stop the injection. Sample were incubated at 25℃ for 6, 24, 48, 72 and 96 hours.

Determination of fruits color and pigments

After injection, tomato fruits are incubated in a transparent plastic container at phytotron. Fruit color was estimated daily through the CIE L*a*b* system using a Chroma Meter CR300 (Konica Minolta) and expressed by the Hue value ($H°_{ab}=tg^{-1}$ (b*/a*). Chlorophyll, β-carotene and lycopene were determined according to the method of Nagata and Yamashita (Nagata and Yamashita, 1992) with slightly modification. Wrap the tube with Aluminum Film during the processing avoid light. Grind tomato fruit pericarp to a fine powder quickly in liquid nitrogen. Take 100 mg powder to a 2.0 ml fresh tube, add 1.5 ml solvent acetone-hexane mixture (4:6), shaking for 30 min. Centrifuge 10,000 g for 2 min at 4℃. Take the supernatant to a new tube and centrifuge again for 2 min. Use a UV-VIS spectrophotometer to determine the absorbance of each sample at 663 nm, 645 nm, 505 nm and 453 nm separately. Calculate the content of chlorophyll and carotenoid use the equation below. Chlorophyll (mg/100ml): 0.671A663+1.671A645; β-carotene (mg/100ml): 0.216A663–1.22A645–0.304A505+0.452A453; Lycopene (mg/100ml): -0.0458A663+0.204A645+0.372A505–0.0806A453. The results were expressed as micrograms of carotenoid per gram of extract.

Measurement of ethylene production

The rate of ethylene produced of whole fruits were measured by individual fruit in an 120 ml air-tight containers for 30 min at 24℃, withdraw 1 ml of the headspace gas and inject into a gas chromatograph 7820A (Agilent Technologies). The unit of ethylene is nmol g^{-1} h^{-1}. The measurement conditions was as follows: the heater temperature is set to 110℃, column flow is at 43 ml min^{-1}, temperature of the oven is at 70℃, FID heater is at 250℃, H_2 flow and air are 40 ml min^{-1} and 350 ml min^{-1} respectively.

Western blot analysis the inhibition of plastid translation

After 48 h incubation at phytotron, whole tomatoes were stored at -80℃ before the following experiment. Grind the tomato pericarp to well powder in liquid nitrogen. Take 300 mg to a 2.0 ml flesh tube extraction buffer (4% SDS, 125 mM Tris-HCl pH6.8) vortex well. Boiled the mixture for 10 min with vortex occasion, centrifuged at RT, 16000 g for 20 min. Transfer the supernatant to a flesh tube and stored. Total protein were separated by SDS-PAGE, transfer to a nitrocellulose membrane, treated with blocking TTBS buffer 20 mM TRIS, 137 mM NaCl, 0.1% (v/v) Tween-20, pH 7.6, containing 2% (w/v) of ECL Advancing Blocking, and subsequently incubated for 1 h with polyclonal antibodies in TTBS. Detection was performed with a peroxidase labelled anti-rabbit antibody (GE Healthcare, NA934), diluted 1:50 000 in TTBS, and the membranes were developed using the ECL Advancing Western blotting detection reagents (GE Healthcare). All the antibodies used at the appropriate dilution for Plastidial photosystem II D1 protein (PsbA/D1, 32 kD, at 1:25 000 dilution), Rubisco large subunit (RbcL, 53 kD, at 1:50 000 dilution), Mitochondrial voltage-dependent anion-selective channel protein I (VDAC1, 29 kD, at 1:5000 dilution), Sucrose phosphate synthase (SPS, 120 kD, at 1:10 000 dilution), Cell wall protein polygalacturonase (PG, 41-43 kD, at 1:5000 dilution) and Vacuolar ATPase (V-ATPase, 26 kD, at 1:5000 dilution). All the antibody bought from Agrisera® except PG generated by lab from recombinant proteins corresponding to X77231 cDNA.

Real-time PCR analysis of expression of genes involved in tomato ripening

RNA Extraction used Plant RNA Reagent kit. Take 100 mg dry frozen tomato pericarp powder and 0.5 mL reagent to a 1.5 mL tube, after 5 min incubated at room temperature, centrifuged at 12000 g for 2 min, Add 0.1mL 5 M NaCl and 0.3 mL chloroform to the supernatant and mix thoroughly. Centrifuged at 4℃, 12000g for 15min, transfer the aqueous to a flesh tube, add equal volume isopropyl alcohol mix well, centrifuged at 4℃, 12 000 g for 15 min, RNA pellets were washed with 75% ethanol and dissolved in RNase-free water, Storage at -80℃.

DNase treatment with RNA: add 0.1 volume 10X DNase I buffer and I μL rDNase to RNA sample mix well and incubate at 37℃ for 60 min, inactivate reaction with 0.1

volume DNase Inactivation Reagent and mix well, incubation for 2 min at RT, centrifuged at 10000 g for 1.5 min and transfer RNA to a fresh tube. For cDNA preparation, add 2 μg template RNA to the master mix reagent (2 uL 10X buffer, 2 uL dNTP Mix, 2 uL oligo dT primer, 1 uL RNase inhibitor, 1uL reverse transcriptase and variable water to volume 20 uL), mix thoroughly and incubated at 37℃ for 60 min. stored the reverse-transcription reaction at -20℃.

To examine the expression of the tomato genes infected by lincomycin, the cDNA samples were used as templates in real-time PCR assay in the presence of a SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers. qPCR reactions were performed as follow: 50 ℃ for 2 min, 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min and one cycle of 95 ℃ for 15s and 60 ℃ for 15s. Relative expression levels were calculated using the ΔΔCt method as described by Lyi et al. (2007). The primer of the checked genes as follow:

Primer	sequence	accession No.
HDR-F	AGTAACACTTCACATCTACAGGAG	solyc01g109300
HDR-R	GTTCTCTTTCTCAACCAACTCAC	
GGPS-F	GCTGTTGGTGTCTTATATCGTG	solyc09g008920
GGPS-R	CTTCTCAATGCCATAAACGCTG	
PSY1-F	GGAAAGCAAACTAATAATGGACGG	Solyc03g031860
PSY1-R	CCACATCATAGACCATCTGTTCC	
PDS-F	GGTCACAAACCGATACTGCT	Solyc03g123760
PDS-R	AAACCAGTCTCGTACCAATCTC	
PTOX-F	GATGAAAGCCTGTCAAACTCAC	solyc11g011990

PTOX-R	CAATCAGCTTGAGGTACGGA	
ZDS-F	AGTGGTTTCTGTCTAAAGGTGG	Solyc01g097810
ZDS-R	ACCGAGCACTCATGTTATCAC	
CRTISO-F	AAGACCCACAGACGATACCT	Solyc10g081650
CRTISO-R	ATCGCCAACACAATATAGACCA	
LCY E-F	CTTACCAGTTCAAGTATCCCGAG	Solyc12g008980
LCY E-R	GCAATATCAGAGCCAGTCCA	
LCY B-F	GTCCACTTCCAGTATTACCTCAG	Solyc04g040190
LCY B-R	TGTCCTTGCCACCATATAACC	
CRTR B2-F	CTTCTTTCCTACGGTTTCTTCCA	Solyc03g007960
CRTR B2-R	CTCTTATGAACCAGTCCATCGT	
ACS1a-F	AAGGTTTATGGAGAAAGTGAGAGG	Solyc08g081550
ACS1a-R	AAAGGCATCACCAGGATCAG	
ACO1-F	ATTGCACAAACAGACGGGAC	Solyc07g049530
ACO1-R	ACTTGTGTACTTTCCTCTGCCT	
OR-F	TTGTGGCATCATTCTCTGGG	Solyc03g093830
OR-R	AGCAAGATATCCTGTTCCAAGAC	
RIN-F	CAACATCTTTCCTCTTACAACCAC	Solyc05g012020
RIN-R	GGTCAAGCTCATTATTCCTCCT	
CNR-F	TGTGAGTTCCATTCAAAGTCTCC	Solyc02g077920
CNR-R	TCCTCTTAGCATCATCAAACTCTG	
PFTF-F	CTTGGAAATGTTGGGCTTGG	Solyc07g055320

PFTF-R	GACATCCTTGAGTTAGAAACACCT	
GUN4-F	CTTGCCATTAACGAATGCTCTG	Solyc06g073290
GUN4-R	CATCTCCATCTTCAACAAATGCTG	
GUN5-F	TCAGGAAACATGCACTAGAACAG	Solyc04g015750
GUN5-R	TGTTGGAAGAGTAAGAACCCGA	
FC1-F	AAAGAAGCAGTGTAGGTGGAG	Solyc10g084140
FC1-R	CGTAGACAATCCAACAGAGCA	
CA1-F	GTCCCACCTTACGATCAGAC	Solyc05g005490
CA1-R	AGTCCTTTAATACCTCCACAACAG	

HDR, hydroxymethylbutenyl diphoshate reductase; GGPS, geranylgeranyl diphosphate synthase; PSY1, phytoene synthase 1; PDS, phytoene desaturase; PTOX, plastid terminal oxidase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCY-E, lycopene ε-cyclase; LCY-B, lycopene β-cyclase; CRTR-B2, β-carotene hydroxylase 2; ACS1a, 1-aminocyclopropane-1-carboxylic acid synthase; ACO1, 1-aminocyclopropane-1-carboxylic acid oxidase; OR, orange; RIN, ripening inhibitor; CNR, Colorless non-ripening; PFTF, plastid fusion and/or translation factor; GUN4, genome uncoupled 4; GUN5, genome uncoupled 5; FC1, ferrochelatase 1; CA1, carbonic anhydrase1.

Result and discussion

Assessment by western blot of lincomycin inhibition of protein translation in plastid

In order to verify that lincomycin was efficient and specific in inhibiting plastid translation, proteins representing different subcellular compartments were analyzed by

immuno-detection. A representative of plastid-translated protein is the plastidial photosystem II D1 (PsbA/D1) protein which is membrane-embedded in the large

photosystem II complex. As shown in figure 1, the abundance of the PsbA/D1 protein is much lower in lincomycin-treated than in control indicating a strong inhibition of translation, thus demonstrating that the lincomycin treatment was efficient. Former experiments had shown the efficiency of lincomycin treatment in inhibiting the PsbA/D1 protein accumulation in plastid (Kim et al. 1994). RbcL is the large subunit of Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) encoded by the plastid genome. Our data show that the amount of the RbcL protein remains essentially unchanged in control and lincomycin-treated samples. This is in agreement with the data of Hill et al. (2011) indicating that there is no significant differences in RbcL level between the samples treated with or without lincomycin in coral nubbins and with the data of Wostrikoff et al. (2012) showing that maize mesophyll protoplasts treated with lincomycin did not exhibit mRNA instability. The RbcL protein was present in stable amounts during the chloroplast-to-chrompoplast transition in bell pepper despite a sharp decrease in expression of the corresponding gene (Kuntz et al. 1989). These data indicate that RbcL is very stable and that the cessation of translation does not affect significantly the amount of protein in short time. The specificity of inhibition of translation in the plastid only was confirmed by analyzing the amount of proteins known to be located in other cell compartments. The voltage-dependent anion channel (VDAC), which plays a central role in apoptosis, is located on the outer membrane of mitochondria (Betaneli et al., 2012). Fig. 1 shows that the expression of the VDAC protein is not affected by lincomycin treatment indicating that lincomycin does not inhibit protein translation in the mitochondria. Similar conclusions can be drawn from the immunodetection of the vacuolar-type H^+-ATPase (V-ATPase) which is a highly conserved evolutionarily ancient enzyme with remarkably diverse functions in eukaryotic organisms (Nelson et al., 2000). The amount of polygalacturonase (PG), a typical cell wall protein (Hadfield and Bennett, 1998) and of sucrose phosphate synthase (SPS), a marker protein for the cytosol (Lunn and Furbank, 1997), are not affected by lincomycin.

Overall, the western blot analysis of the different proteins indicated that the antibiotic lincomycin treatment was efficient and that it inhibited specifically the translation of proteins in the plastids of tomato fruits.

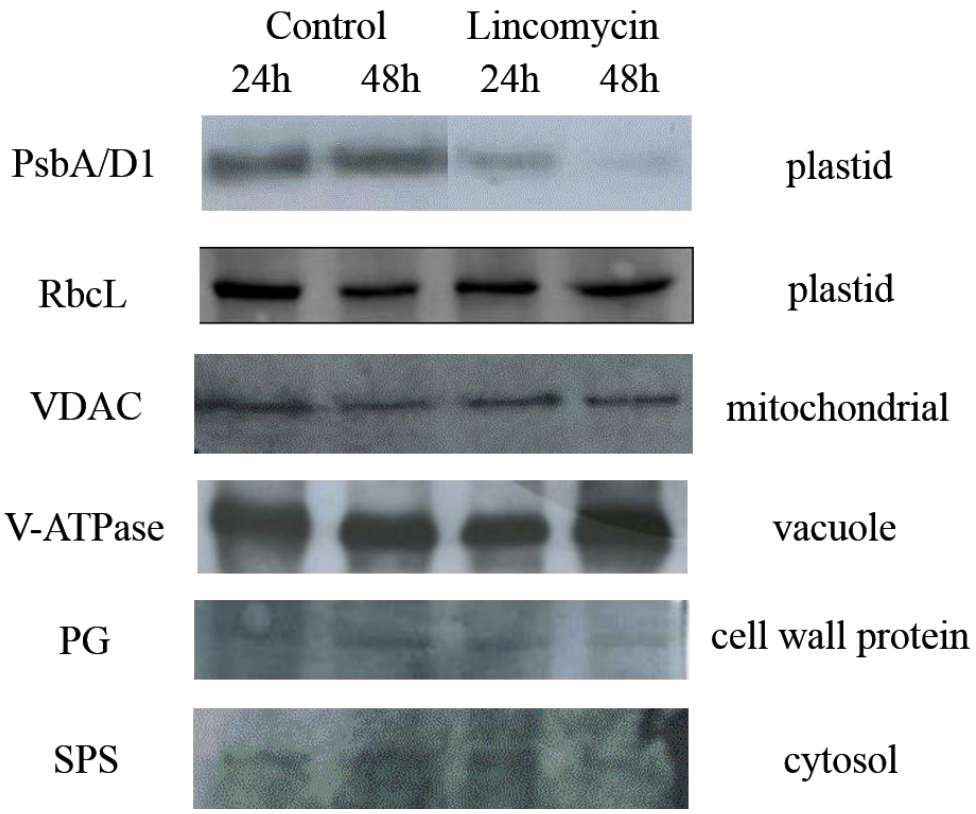

Fig.1 Western blots showing the effects of lincomycin on the accumulation of proteins located in various sub-cellular compartments. (PsbA/D1: plastidial photosystem II D1; RbcL: rubisco large subunit; VDAC: voltage-dependent anion channel; V-ATPase: vacuolar-type H+-ATPase; PG: polygalacturonase; SPS: sucrose phosphate synthase)

Effects of lincomycin on the changes in color and pigments of ripening tomatoes

After injection of lincomycin in breaker tomatoes as described in “Material and Methods”, the changes in color were assessed by chromametry and the content of chlorophyll and carotenoids measured by spectrophotometry. Figure 2A shows visible differences in the changes in color between control (infiltrated with buffer only) and

lincomycin-infiltrated tomatoes. The lincomycin treatment significantly delayed the changes of color from green to red as compared to control fruit although the variations between samples is elevated.

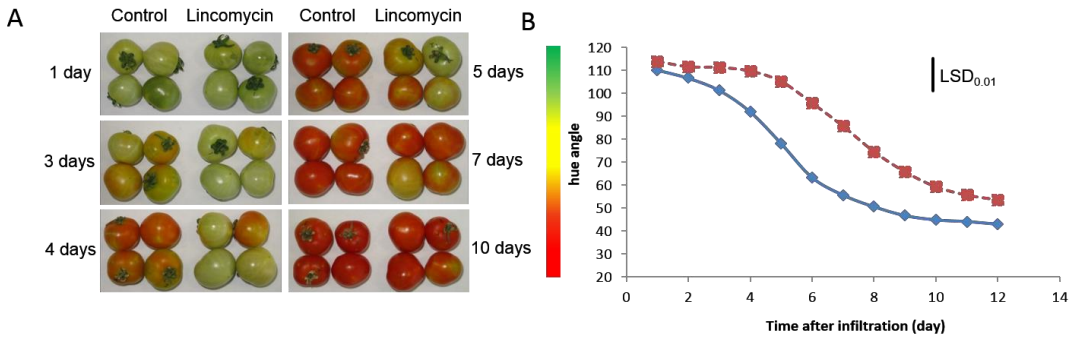

Fig. 2 Effects of lincomycin on color change of tomato fruits. Pictures were taken of the external color (A); Hue angle value changes after lincomycin treated (B). Control injected with buffer only (◆), treated with 2 mM lincomycin (■). n = 6, LSD bar calculated at the 1% risk.

An evaluation of the Hue angle, a main property of color, has been performed by chromametry (Fig. 2B). The Hue angle value decreased significantly faster in control than in lincomycin-treated fruit thus confirming a lower rate of tomato fruit color change from green to red. An evaluation of the pigments extracted from the fruit indicated that the delay of ripening mainly due to a low rate accumulation of lycopene (Fig.3A). However here again, although differences remain significant, there is often a large variation between samples, for example 100 hours after infiltration in lincomycin-treated fruit. Interestingly, the decrease of chlorophyll and the increase in β-carotene were similar in control and lincomycin-treated fruit (Fig. 3B and 3C). Therefore, the delay of color change mainly depends on the lycopene synthesis and accumulation. Lycopene and β-carotenes are the main metabolites in the carotenoid synthesis pathway.

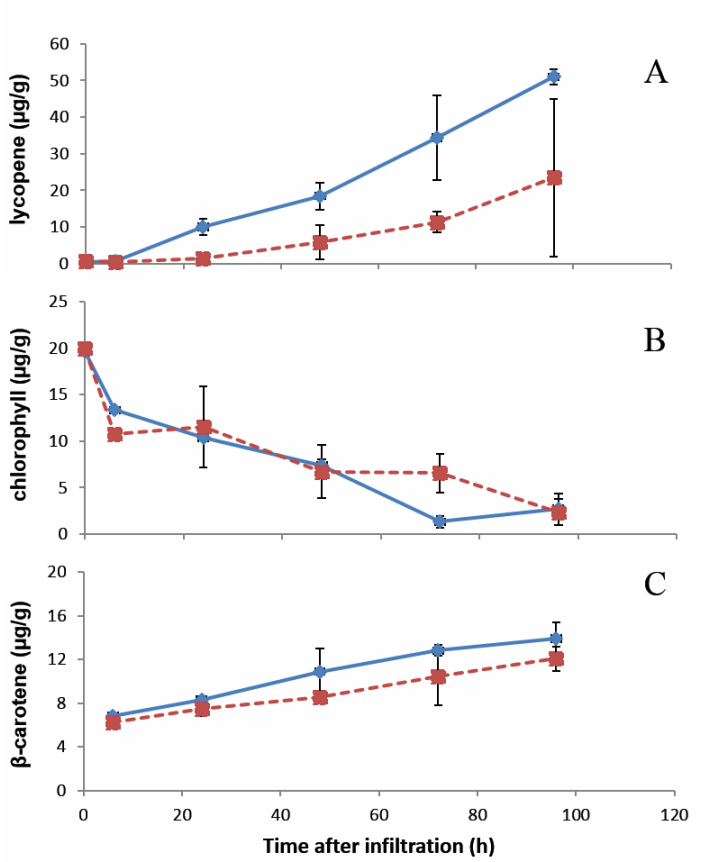

Fig. 3 Kinetics of pigments after infiltration with lincomycin. Control injected with buffer only (◆), treated with 2 mM lincomycin (■). n = 6, error bars show SE.

Effects of lincomycin on ethylene production of the tomatoes

To determine whether ethylene was involved in the response to the lincomycin treatment, ethylene production was evaluated by gas chromatography. Six biological replicates were performed and the data were combined and analyzed. Figure 4 shows that no significant differences could be observed between control and lincomycin-treated fruit in terms of ethylene production. It also shows high standard errors for samples taken at all times after infiltration. Therefore, these preliminary result suggest that lincomycin has no effect on the rate of ethylene production in tomato.

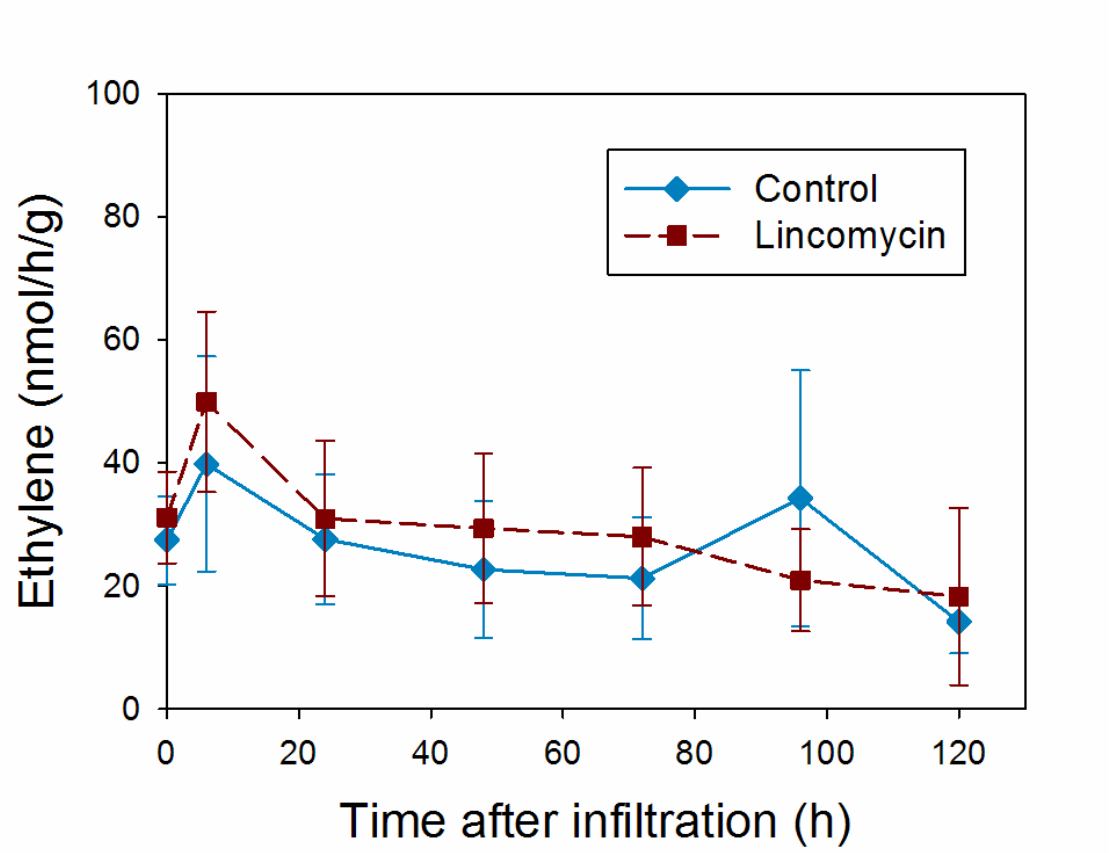

Fig. 4 Ethylene production changes after lincomycin treated. Control injected with buffer only and treated with 2mM lincomycin . n = 6, error bars show SE.

Effects of lincomycin on the expression of carotenoid biosynthesis genes.

During tomato fruit ripening, carotenoid accumulate in the skin and flesh as the fruit turns red. Since we have observed previously a delay in the accumulation of carotenoids upon lincomycin treatment, we have carried out a transcriptional analysis of genes involved in the synthesis of carotenoids. A scheme of the carotenoid biosynthetic pathway is given in figure 5A. A qPCR analysis of genes of the downstream MEP pathway (HDR and GGPS) and of carotenoid biosynthesis (PSY1, PDS, PTOX, ZDS, CRTISO, LCY-E, LCY-B and CRTR-B2) has been performed. The data presented in figure 5B indicate that most of the transcripts remain stable except those of PSY1, PTOX, and ZDS which have significant increase and LCY-B undergoes a decrease as compared to control.

Phytoene synthase plays an important role in the synthesis of phytoene and it catalyzes the first committed reaction in carotenogenesis (Maass et al., 2009; Cazzonelli and Pogson, 2010). The PTOX is a plastoquinol-O_2 oxidoreductase that regenerates the reduced plastoquinone formed during phytoene and ζ-carotene desaturation (Ruiz-sola et al., 2012). The absence of PTOX result in tomato ghost phenotype with green and bleached sectors leaves (chloroplast) and carotenoid deficient ripe fruits (chromoplast) (Josse et al., 2000; Shahbazi et al., 2007). ZDS is a ζ-carotene desaturase involved in this pathway. PSY, PTOX and ZDS exhibit higher expression after lincomycin treatment indicating that lycopene could accumulate at a high level during development. However, the contents of lycopene of lincomycin-treated fruits were lower than control as shown in figure 3A. One hypothesis is that the provision of precursors by the upsteram steps is insufficient so that the higher expression of genes of the carotenoid pathway is useless for carotenoid accumulation. It may also happen that the higher expression of some genes, such as phytoene synthase is not accompanied by an increased level of the protein. A discrepency between enzyme activty and gene expression of phytoene synthase has already been observed in tomato fruit sumitted to phytochrome regulation of phytoene synthase activity by red and far red light (Schofield and Paliyath, 2005) suggesting that PSY may be subject to post-translational regulation. Further work is needed to test whether this happens in our conditions upon lincomycin treatment.

Concerning the LCY-B gene which is reposnsible for the accumulation of β-carotene (Ampomah-Dwamena et al., 2009) we observed a down-regulation after lincomycin treatment. This is not consistent with a lower accumulation of lycopene. It could happen that the lower accumulation of lycopene upon lincomycin treatment arises from a lower provion of carotenoid precursors. For this reason, we have analysed key enzymes of the MEP pathway that supplies Geranylgeranyl diphosphate (GGPP) to the carotenoid pathway. Hydroxymethylbutenyl diphoshate reductase (HDR) and geranylgeranyl diphosphate synthase (GGPS) are the two key enzyme involved in MEP pathway (Botella-Pavia et al., 2004). Our data show that there is no significant change in the expression of the two genes after infiltration with lincomycin (Fig. 5B) indicating that the provision of precursors is probably not responsible for the lower accumulation of lycopene in lincomycin-treated fruit. In conclusion, our data on gene expression cannot

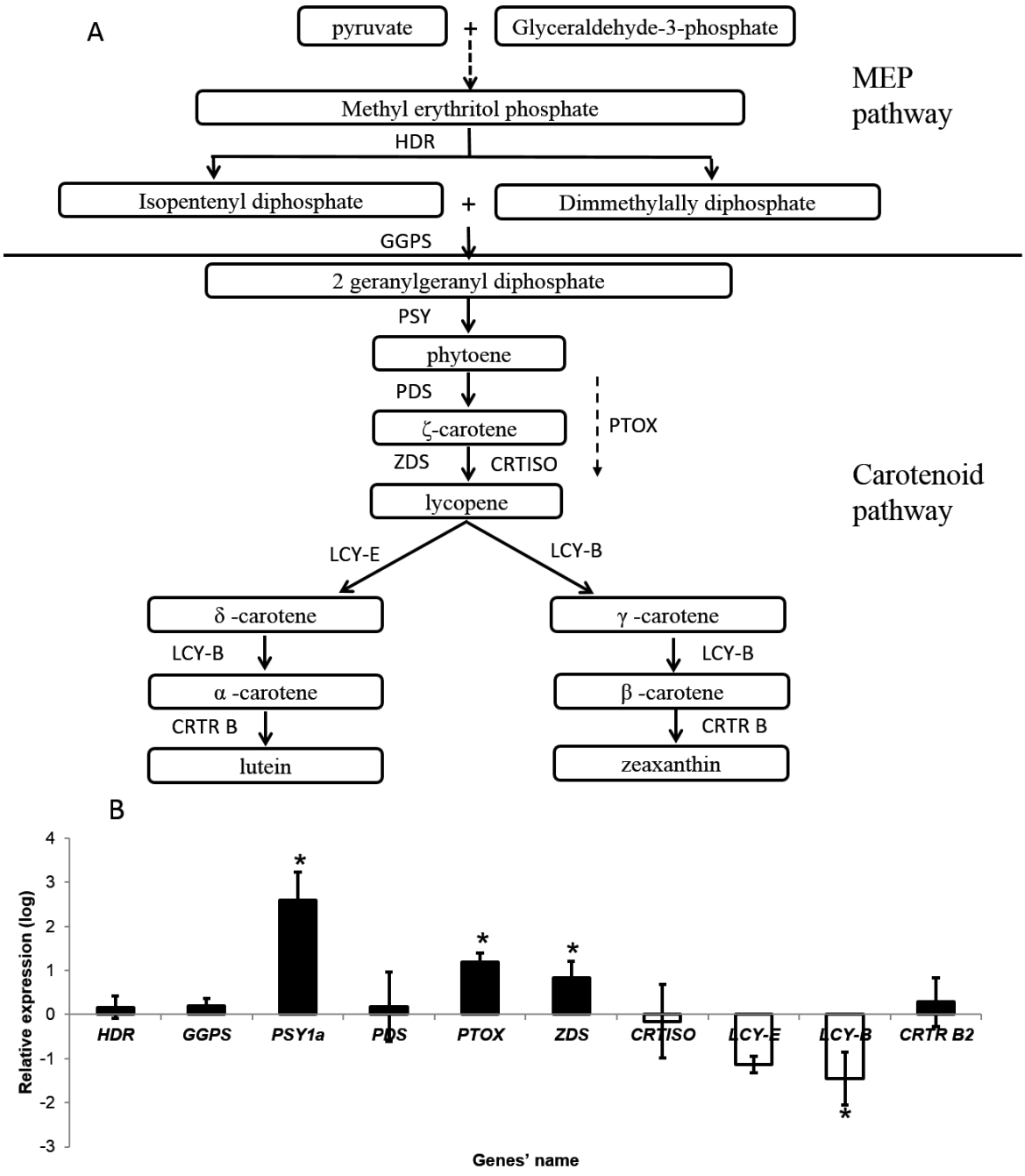

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Fig. 5 Enzymes involved in the carotenoid biosynthesis. The pathway of carotenoid synthesis and the downstream of MEP pathway (A), Effect of lincomycin on the expression of genes in carotenoid pathway (B). Controls were set at 0, n = 3, error bars show SE. (HDR: hydroxymethylbutenyl diphoshate reductase; GGPS: geranylgeranyl diphosphate synthase; PSY1: phytoene synthase 1; PDS: phytoene desaturase; PTOX: plastid terminal oxidase; ZDS: ζ-carotene desaturase; CRTISO: carotenoid isomerase; LCY-E: lycopene ε-cyclase; LCY-B: lycopene β-cyclase; CRTR-B2: β-carotene hydroxylase 2)

explain the effect of lincomycin on the reduction of accumulation of lycopene.

Effects of lincomycin on the expression of ethylene biosynthesis genes.

ACS1a and ACO1 participating in the ethylene biosynthesis are up-regulated after lincomycin treatment (Fig. 6). ACS1a is encoded by a multigene family. With lincomycin treated, ACS1a as well as the ACO1 has a higher expression than control. Expression of ACS1a is decreasing during the development of tomato fruit (Barry et al., 2000) and ACO1 transcripts increase during ripening (Nakatsuka et al., 1998). Our former ethylene assay indicated that ethylene production was not different between control and lincomycin-treated fruits although the transcripts of both ACS1a and ACO1 are increased. Knowing that ACS1 is negatively and ACO1 positively regulated by ethylene we cannot suspect an indirect effect of lincomycin on the activity of the receptor(s) otherwise the expression of the two genes would be inversely affected. In the present state of our research we don't have any clear explanation for the up-regulation of ACS1 and ACO1 upon lincomycin treatment while ethylene production remains unchanged.

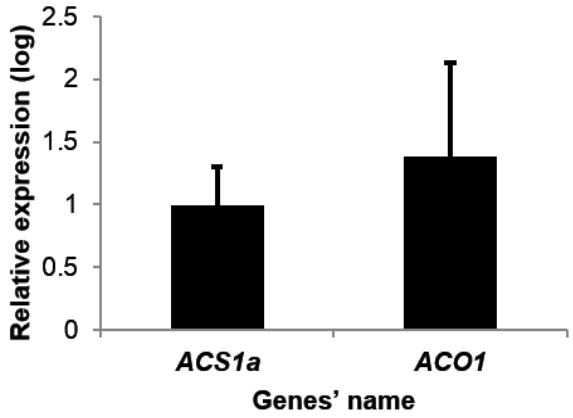

Fig. 6 Effect of lincomycin on the expression of genes involved in the ethylene biosynthesis. controls were set at 0, n = 3, error bars show SE. (ACS1A: 1-aminocyclopropane-1-carboxylic acid synthase; ACO1: 1-aminocyclopropane-1-carboxylic acid oxidase)

Effects of lincomycin on the expression of regulatory genes involved in fruit ripening and chromoplast differentiation.

Several transcription factors have been described as regulating fruit ripening apart from the ethylene signaling or biosynthetic pathways. One of them is a MADS box named "ripening inhibitor" (rin). The loss-of-function rin mutant (Zhu et al., 2007) shows

inhibition of color development, softening, aroma production etc. In our experiments, the expression of the *RIN* gene was depressed significantly by lincomycin treatment (Fig. 7). This could explain the delay of lycopene accumulation and fruit ripening. Another transcription factor CNR (colorless non-ripening) involved in color changes has been studied shows no change in expression upon lincomycin treatment (Fig. 7).

The expression of two other genes involved in the differentiation of plastids has been considered. The DNAJ-type gene Or, involved in the differentiation of chromoplasts shows reduced expression in lincomycin-treated fruit (Fig. 7). Only a mutation of the *Or* gene results in the accumulation of carotenoids. The RNAi knock-down of the Or protein does not cause any accumulation of carotenoids (Lu et al., 2006) suggesting that wild-type Or is most likely not a loss-of-function mutant. In our experiments, lincomycin treatment induces a significant decrease in Or transcripts (Fig. 7), while lycopene accumulates more than in control fruit. In view of this data, we cannot provide a rational explanation for the role of the Or gene in the phenotype of lincomycin-treated fruit.

PFTF (plastid fusion and/or translation factor) has been involved in chromoplast differentiation in red pepper (Hugueney et al., 1995). The qPCR results (Fig. 7) show that gene expression was slightly, but not statistically decreased after lincomycin treatment indicating that this gene cannot be responsible for the effects of lincomycin on the higher accumulation of carotenoids.

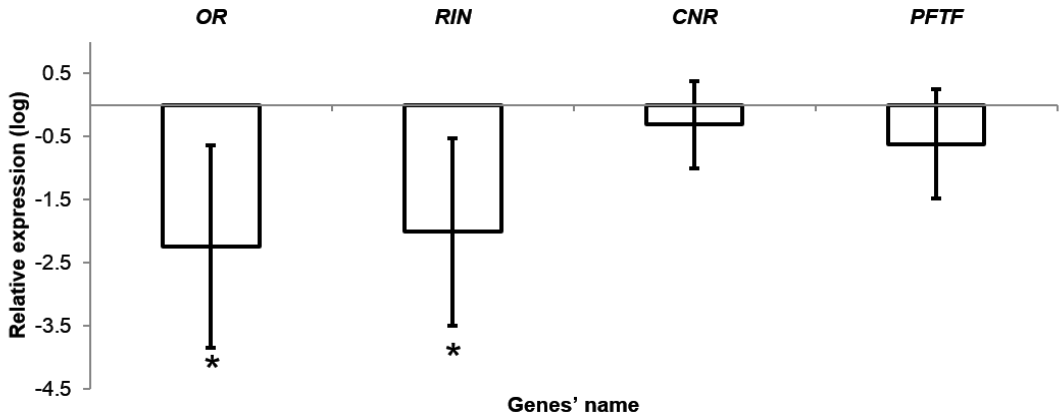

Fig. 7 Effect of lincomycin on the expression of regulatory genes in fruit ripening. controls were set at 0, n = 3, error bars show SE. (OR: orange; RIN: ripening inhibitor; CNR: colorless non-ripening; PFTF: plastid fusion and/or translation factor)

Effects of lincomycin on the expression of genes suspected to participate in the plastid-to-nucleus signaling.

Gun (Genomes uncoupled) genes are involved in the chlorophyll biosynthesis pathway and ferrochelatase 1 (FC1) is the enzyme that participates in heme biosynthesis within the branch of tetrapyrrole biosynthesis. The phenotype of Gun mutants varies from

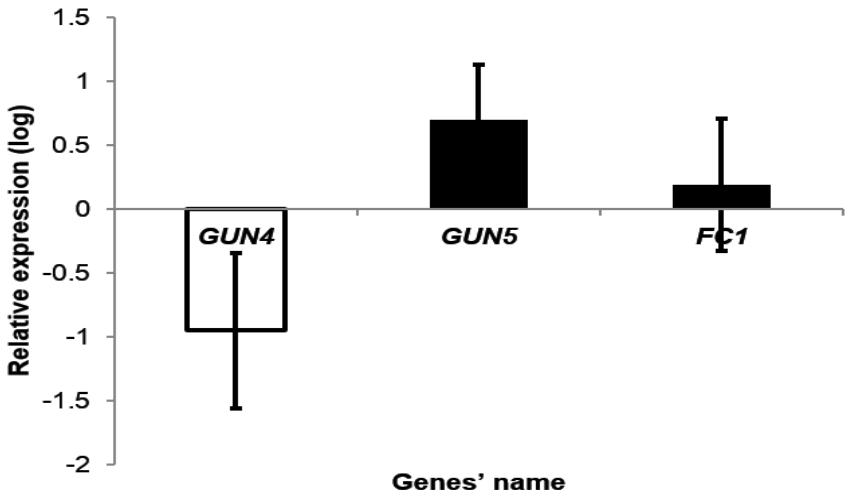

Fig. 8 Effect of lincomycin on the expression of genes suspected to participate in the plastid-to-nucleus signaling. controls were set at 0, n = 3, error bars show SE. (GUN4: genome uncoupled 4; GUN5: genome uncoupled 5; FC1: ferrochrlatase 1)

pale yellowish to undistinguishable from wild-type (Surpin et al., 2002). The Gun4, Gun5 mutants demonstrated that reduced Mg-Proto-IX accumulation under NF treatment causes down-regulation expression of Lhcb (light harvesting complex II) in Arabidopsis (Strand et al., 2003). Gun4 was found to encode an activator of the Mg-chelatase and gun5 was affected in CHL-H, a subunit of Mg-chelatase (Dietzel et al., 2009). Gun4, Gun5 and ferrochelatase1 were analyzed by qPCR. The transcripts of gun4 with a lower level than control, but gun5 has an induced expression. Gun4 as an activator involved in the tetrapyrrole biosynthesis binding either the substrate proto-IX or the product of the chelation reaction, Mg-Proto-IX. But the maximum Mg-chelatase activity requires pre-activation of ChlH with GUN4, Mg^{2+} and proto-IX in vitro of Oryza sativa (Zhou et al., 2012). Gun4 transcripts decreased by lincomycin treated implied that activity of Mg-chelatase is depressed. The consequence is that chlorophyll synthesis was reduced. As

show in Fig. 3B, the decreasing of chlorophylls is similar between control and treated samples. One of the possibility is that almost all the enzymes involved in the chlorophyll synthesis are decreasing or undetectable with tomato fruit ripening (Barsan et al., 2012). Exception is ferrochelatase1 which is located in the heme branch of tetrapyrrole pathway. Recently, Woodson et al. (2011) present a new Arabidopsis mutant, gun6-1D, with similar phenotype of the other gun mutants. The ferrochelatase1 is overexpressed in this gun6-1D mutant and which demonstrate that increased flux through the heme branch of the plastid tetrapyrrole pathway increases PhANGs expression. Our data show that ferrochelatase1 is slightly induced by lincomycin. That indicated there will be signals produced for the communication of plastid to nucleus.

Effects of lincomycin on the expression of CA1, a gene responsive to lincomycin.

Carbonic anhydrase 1 (CA1) is one of the most down-regulated genes upon lincomycin treatment in Arabidopsis seedlings (Cottage et al., 2008). Figure 9 shows that the CA1 gene is not significantly down-regulated in our conditions. The possibility is that different tissues have different sensitivity and responsiveness to lincomycin.

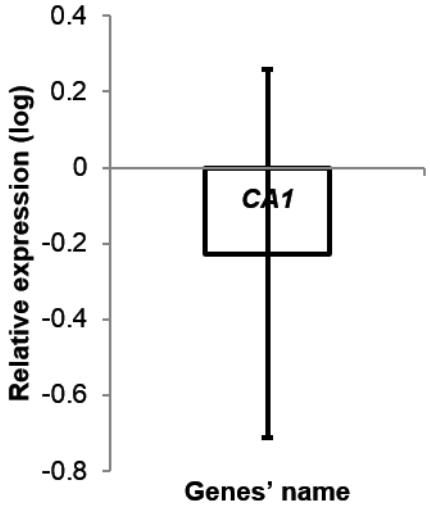

Fig. 9 Effect of lincomycin on the expression of carbonic anhydrase1 (CA1), a gene responsive to lincomycin. Controls were set at 0, n = 3, error bars show SE

Conclusion

This set of results shows that the plastidial translation may have impact on the fruit ripening process and nuclear gene expression. One of the first and most important effects of inhibiting plastid translation is the lowering of carotenoid accumulation which could be interpreted as a delay in the fruit ripening process. However more experiments would be necessary to determine whether other aspects of fruit ripening are affected. In terms of gene expression, inconstant results have been obtained since all genes upstream of lycopene in the carotenoid biosynthetic pathway exhibit higher expression upon lincomycin treatment. Also, the lower production of carotenoids in lincomycin-treated fruit could not be related to a lower expression of genes involved in the provision of precursors. The enhancement of expression of the ethylene biosynthesis genes is also inconsistent with the stable ethylene production. More work is needed to detect whether any discrepancy exist between gene expression and the level of the corresponding protein, as suggested in some occasions with the phytoene synthase gene. The expression of other genes related to various cellular signaling pathways and transcription factors were also affected by lincomycin, such as Or, RIN and GUN, but no clear scheme of their participation in the phenotype generated by lincomycin could be presented. We have always observed high variations between samples giving high error standards. This may be due to the fact that the infiltration of lincomycin in not homogenous within the fruit and from one fruit to another. Anyhow, this preliminary set of results shows that plastidial translation has an impact on fruit ripening, part of which may be due to retrograde signaling, but further work is required to provide a picture of which target gene is affected.

General conclusion

The ripening of fruits is a very complex process comprising many metabolic and biochemical events. As a model for climacteric fruit, tomato has been largely used for studying the physiological and molecular basis of the ripening process. Color is one of the most important features during tomato fruit ripening. The green color which is accompanying tomato fruit development starts to disappear at the breaker stage with the development of a yellow color to be totally absent at the red stage. The changes of color are due to the decrease of chlorophylls and the accumulation of carotenoids. These changes take place in the plastids and correspond to the transition from chloroplasts to chromoplasts. Early studies have provided a detailed structural description of this transition using microscopy techniques (Rosso, 1968; Harris and Spurr, 1969) although recent techniques of confocal microscopy can bring novel information. In the present thesis we have used confocal microscopy coupled to spectrometry to monitor the chloroplast-to-chromoplast transition by evaluating the chlorophyll and carotenoid content of single plastids within live tissues. Our results give evidence that each chromoplasts derive from a single chloroplast and that differentiation of plastids then ceased in ripening fruits. Plastids have been isolated at different stages of tomato fruit ripening, mature green (MG), breaker (B) and red (R), and observed by confocal microscopy. As expected chloroplast of mature-green fruit emitted fluorescence due almost exclusively to chlorophyll and chromoplast of red fruit to carotenoids. Interestingly, we have been able to isolate intermediate plastids from breaker stage fruit harboring both chlorophyll and carotenoids appearing in orange color in the overlay picture. Real-time in-situ recording of the live tissue showed that the transition was rather synchronous within one cell but not at the tissue level. The real-time monitoring of the transition presented in a video revealed that all the chromoplasts derived from pre- existing chloroplasts in tomato mesocarp tissues. The confocal microscopy characterization of the plastids at different stages of development has been highly useful for the selection of plastid fractions for proteomic analysis.

Following the early microscopy observations, studies have been dedicated to the understanding of the biochemical and molecular events underlying chromoplastogenesis. A description of the changes occurring in number of metabolic pathways has been performed with special emphasis to the biosynthesis of carotenoids (Camara et al., 1995). More recently a global characterization of chromoplasts from different species has been performed using high throughput technologies (Siddique et al., 2006; Barsan et al., 2010; Zeng et al., 2011), but the overall changes occurring during the chloroplast-to-chromoplast transition have been scarcely studied by proteomic or transcriptomic approaches (Kahlau and Bock, 2008). In the present thesis we have used a proteomic approach for studying the changes in protein abundance during chloroplast-to-chromoplast transition. For this purpose plastids have been isolated at three stages of differentiation and the corresponding proteins were analyzed by proteomics. A total of 1932 proteins have been identified after curation. The curation procedure is generally performed after proteomic analysis. It consisted in discarding proteins that were not predicted to be plastid localized by 3 predicting softwares and not identified previously in plastid databanks. After curation, 1529 proteins could be quantified by spectral counting. Among 87 proteins predicted to be encoded by the plastid genome, 47 were encountered in our study and the abundance of 32 of them is presented for all three stages of plastid development. The accuracy of our quantification procedure was tested in two ways. First we have compared our data with previous data on the whole tomato fruit proteome (Rocco et al., 2006; Faurobert et al., 2007) and observed that, for proteins identified in both cases, the pattern of evolution during fruit ripening was very similar. Second, we have performed an immuno-detection for a number of proteins and have shown that the changes in abundance of the proteins detected by this technique were identical with proteomic data. The proteomic analysis revealed several metabolic features of the transition. As expected there was a shift of metabolism corresponding to a decrease of proteins involved in photosynthesis and an increase in proteins involved in carotenoid biosynthesis. One striking feature, however, is that the large subunit of Rubisco (RBCL), a protein typically abundant in photosynthetic plastids, although continuously decreasing in abundance, remained present at significant levels in red plastids. On the contrary, some proteins PSAD of PSI and PSBA/D of PSII involved in photosynthesis were totally

undetectable at the R stage after a strong decrease in abundance between the MG and B stages. A number of proteins participating in the buildup of thylakoids have been encountered that decrease in abundance during the chloroplast-to-chromoplast transition, accounting for the dismantling of the thylakoids during chromoplastogenesis. A dnaj-like chaperone participating in the differentiation of plastids and corresponding to the product of the Or gene that controls chromoplast differentiation and carotenoid accumulation was encountered. It was more abundant at the breaker stage than at the green stage and was not detected at the R stage. Two nuclear-encoded forms of filamenting temperature-sensitive mutant Z play a major role in the initiation and progression of plastid division in plant cells. They are both present at MG and B stages, but FtsZ1 was absent at the R stage in agreement with the cessation of plastid division. Altogether these data indicate that the major metabolic shifts occurring during chromoplastogenesis are associated with the loss of the machinery involved in the synthesis of thylakoids and in plastid division but also with the up-regulation of elements regulating the differentiation process such as the Or protein.

All this changes are regulated by the nucleus and plastid genes. The coordination between plastids and nucleus gene expression plays an essential role during plastids differentiation. Even a slight perturbation can affect the expression of the two genomes. We have used the antibiotic lincomycin for inhibiting the translation of plastid and to test if the expression of nuclear genes was affected. After lincomycin treatment, the color change of the tomato was significantly delayed. The preliminary results indicate that inhibiting protein translation in the plastids affects fruit ripening by reducing the accumulation of carotenoids. The expression of several nuclear genes has been affected but a clear relationship with the altered ripening phenotype could not be established. These data suggest that a plastid to nucleus signaling may operate during the chloroplast-to-chromoplast transition, but more work is needed to elucidate the elements of such signaling.

Altogether, we present here a work on metabolic and molecular events occurred during transition of chloroplast-to-chromoplast. This gives us a new insights into the process of tomato fruit ripening.

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Abstract

One of the most important phenomenons occurring during tomato fruit ripening is the color change from green to red. This change takes place in the plastids and corresponds to the differentiation of photosynthetic plastids, chloroplasts, into non photosynthetic plastids that accumulate carotenoids, chromoplasts. In this thesis we first present a bibliographic introduction reviewing the state of the art in the field of chloroplast to chromoplast transition and describing the structural and physiological changes occurring during the transition. Then, in the first chapter we present an *in situ* real-time recording of pigment fluorescence on live tomato fruit slices at three ripening stages. By viewing individual plastids it was possible to show that the chloroplast to chromoplast transition was synchronous for all plastids of a single cell and that all chromoplasts derived from pre-existing chloroplasts. In chapter two, a quantitative proteomic approach of the chloroplast-to-chromoplast transition is presented that identifies differentially expressed proteins. Stringent curation and processing of the data identified 1932 proteins among which 1529 were quantified by spectral counting. The quantification procedures have been subsequently validated by immune-blot evaluation of some proteins. Chromoplastogenesis appears to comprise major metabolic shifts (decrease in abundance of proteins of light reactions and CHO metabolism and increase in terpenoid biosynthesis and stress-related protein) that are coupled to the disruption of the thylakoid and photosystems biogenesis machinery, elevated energy production components and loss of plastid division machinery. In the last chapter, we have used lincomycin, a specific inhibitor of protein translation within the plastids, in order to study the effects on fruit ripening and on the expression of some ripening-related nuclear genes. Preliminary results indicate that inhibiting protein translation in the plastids affects fruit ripening by reducing the accumulation of carotenoids. The expression of several nuclear genes has been affected but a clear relationship with the altered ripening phenotype could not be established.

Altogether, our work gives new insights on the chromoplast differentiation process and provides novel resource data on the plastid proteome.

Résumé

L'un des phénomènes les plus importants survenus pendant la maturation du fruit de tomate est le changement de couleur du vert au rouge. Ce changement a lieu dans les plastes et correspond à la différenciation des plastes photosynthétiques, les chloroplastes, en plastes non-photosynthétiques qui accumulent des caroténoïdes, les chromoplastes. Dans cette thèse, nous présentons d'abord une introduction bibliographique sur le domaine de la transition chloroplaste-chromoplaste, en décrivant les modifications structurales et physiologiques qui se produisent pendant la transition. Puis, dans le premier chapitre, nous présentons des observations microscopiques de plastes isolés à trois stades de mûrissement, puis des enregistrements en temps réel de la fluorescence des pigments sur les tranches de fruits de tomate. Il a été possible de montrer que la transition chloroplaste-chromoplaste était synchrone pour tous les plastes d'une seule cellule et que tous les chromoplastes proviennent de chloroplastes préexistants. Dans le deuxième chapitre, une approche protéomique quantitative de la transition chloroplaste-chromoplaste est présentée, pour identifier les protéines différentiellement exprimées. Le traitement des données a identifié 1932 protéines parmi lesquelles 1529 ont été quantifiées par spectrométrie de masse. Les procédures de quantification ont ensuite été validées par WESTERN blot de certaines protéines. La chromoplastogénèse comprend les changements métaboliques suivants : diminution de l'abondance des protéines de réaction à la lumière et du métabolisme des CHO, et l'augmentation de la biosynthèse des terpénoïdes et des protéines de stress. Ces changements sont couplés à la rupture de la biogenèse des thylakoïdes, des photosystèmes et des composants de production d'énergie, et l’arrêt de la division des plastes. Dans le dernier chapitre nous avons utilisé la lincomycine, un inhibiteur spécifique de la traduction à l’intérieur des plastes, afin d’étudier les effets sur la maturation des fruits et sur l’expression de gènes nucléaires impliqués dans la maturation. Les résultats préliminaires indiquent que l’inhibition de la traduction des protéines dans les plastes affecte la maturation du fruit en réduisant l’accumulation de caroténoïdes. L’expression de plusieurs gènes nucléaires a été modifiée mais une relation claire avec le phénotype altéré de maturation n’a pas pu être établie.

Au total, notre travail donne de nouveaux aperçus sur le processus de différenciation chromoplaste et fournit des données nouvelles ressources sur le protéome plaste.